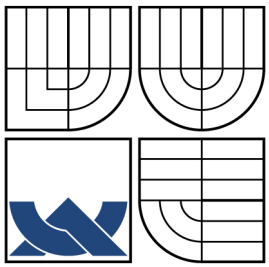

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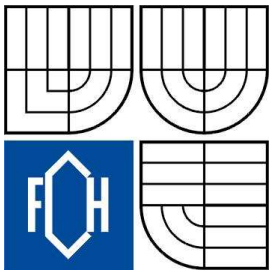

FACULTY OF CHEMISTRY
INSTITUTE OF CHEMISTRY AND TECHNOLOGY OF ENVIRONMENTAL PROTECTION

MOLECULAR STUDY OF INTRACELLULAR CHANGES AS RESPONSE OF MICROORGANISMS TO ENVIRONMENT

PHD THESIS

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ABSTRACT

In nature yeast cells live in a rapidly changing environment. In order to survive constant fluctuations in their external surroundings cells quickly and effectively adapt their internal systems to meet the challenges of each new environment. One aspect of this cellular adaptation is the reorganization of genomic expression to the program required for growth in each environment. This genomic reorganization results in altered cell's metabolome and physiology. The molecular responses elicited by the cells dictate whether the organism adapts, survives or, if injured beyond repair, undergoes death.

In this work, genomic, proteomic and metabolic profiles of some carotenogenic yeasts grown in optimal and stress conditions were compared. To increase the yield of carotenoid pigments, several types of environmental changes as well as nutrition stress were applied. Yeasts were cultivated under osmotic, oxidative and metal stress, on different carbon sources or on various waste materials (whey, potato extract). Some chemical and UV mutants were also prepared and tested. Changes on yeast metabolome level were studied by RP-HPLC/MS technique used to carotenoid analysis.

Another possibility to study stress response is preparation of transformed cells with deletion of definite genes. For setup, the method was first optimized for fission yeasts *Schizosaccharomyces pombe*. Technique is based on knockout constructs that contain regions homologous to the target gene cloned into vectors carrying dominant drug-resistance markers. As transformation vector plasmid pCloneKanmx confering resistance to geneticine was used. The vector and constructs were digested by relevant restriction endonucleases, ligated, amplified in *E.coli* and transformed into the yeast *Schizosaccharomyces pombe* sp286. Positive transformants were selected according to resistance to geneticine and checking PCR products. By analysis of transformants proteins required for meiotic chromosome segregation were identified.

KEYWORDS
environmental stress, environmental stress response, carotenogenic yeasts, carotenoids

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DECLARATION

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student's signature

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1. INDEX TO ABBREVIATIONS

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
mRNA	messenger ribonucleic acid
ER	endoplasmic reticulum
ORF	open reading frame
ROS	reactive oxygen species
ESR	environmental stress response
HSP	heat shock protein
SC, S. cerevisiae	*Saccharomyces cerevisiae*
SP, Sz. pombe	*Schizosaccharomyces pombe*
RG, R. glutinis	*Rhodotorula glutinis*
RR, R. rubra	*Rhodotorula rubra*
RA, R. aurantiaca	*Rhodotorula aurantiaca*
SR, S. roseus	*Sporobolomyces roseus*
Ss, S. shibatanus	*Sporobolomyces shibatanus*
CC, C. capitatum	*Cystofilobasidium capitatum*
wt1	yeast cultivation on conventional glucose medium; 1st series
wt2	yeast cultivation on conventional glucose medium; 2nd series
wt3	yeast cultivation on conventional glucose medium; 3rd series
WL1	yeast cultivation on media with whey lyophilized; 1st series
WL2	yeast cultivation on media with whey lyophilized; 2nd series
WL3	yeast cultivation on media with whey lyophilized; 3rd series
WLA1	yeast cultivation on media with processed/acidified lyophilized whey; 1st series
WLA2	yeast cultivation on media with processed/acidified lyophilized whey; 2nd series
WLA3	yeast cultivation on media with processed/acidified lyophilized whey; 3rd series
Wl1	yeast cultivation on liquid whey; 1st series
Wl2	yeast cultivation on liquid whey; 2nd series
Wl3	yeast cultivation on liquid whey; 3rd series
PE1	yeast cultivation on media with potato extract; 1st series
PE2	yeast cultivation on media with potato extract; 2nd series
PE3	yeast cultivation on media with potato extract; 3rd series
wt/p1	yeast cultivation on conventional glucose medium in potato experiment; 1st series
wt/p2	yeast cultivation on conventional glucose medium in potato experiment; 2nd series
G-PE1	yeast cultivation on media with glucose + potato extract; 1st series
G-PE2	yeast cultivation on media with glucose + potato extract; 2nd series
PE1	yeast cultivation on media with potato extract; 1st series
PE2	yeast cultivation on media with potato extract; 2nd series
G-PP1	yeast cultivation on media with glucose + potato peels; 1st series
G-PP2	yeast cultivation on media with glucose + potato peels; 2nd series
PP1	yeast cultivation on media with potato peels; 1st series
PP2	yeast cultivation on media with potatopeels; 2nd series

wt/a1	yeast cultivation on conventional glucose medium in apple experiment; 1st series
wt/a2	yeast cultivation on conventional glucose medium in apple experiment; 2nd series
AF1	yeast cultivation on media with apple fiber; 1st series
AF2	yeast cultivation on media with apple fiber; 2nd series
G-AF1	yeast cultivation on media with glucose + apple fiber; 1st series
G-AF2	yeast cultivation on media with glucose + apple fiber; 2nd series
CA1	yeast cultivation on media with crushed apple; 1st series
CA2	yeast cultivation on media with crushed apple; 2nd series
AP1	yeast cultivation on media with apple peels; 1st series
AP2	yeast cultivation on media with apple peels; 2nd series
wt/c	yeast cultivation on conventional glucose medium in experiment with cereal substrates;
Meal	yeast cultivation on media with wheatmeal;
Pastes	yeast cultivation on media with pastes;
Pastes/E	yeast cultivation on media with pastes pretreated by hydrolytic enzymes;
AF	yeast cultivation on media with apple fiber;
AF/E	yeast cultivation on media with apple fiber pretreated by hydrolytic enzymes;
WGF	yeast cultivation on media with whole-grain frumenty;
WGF	yeast cultivation on media with whole-grain frumenty pretreated by hydrolytic enzymes;
Grains	yeast cultivation on media with grains;
Grains/E	yeast cultivation on media with grains pretreated by hydrolytic enzymes;
Bran	yeast cultivation on media with wheatbran;
Bran/E	yeast cultivation on media with wheatbran pretreated by hydrolytic enzymes;
CDW	cell dry weight
RP - HPLC	reverse phased - high-performance liquid chromatography
PDA	photodiode array
MS	mass spectrometry
ESI	electrospray ionization
PI ESI-MS	positive ion electrospray ionisation mass spectrometry
DHE	dihydroethidium
H_2-DCF-DA	2‘,7‘-dichlorodihydrofluorescein diacetate
PFGE	pulsed field gel electrophoresis
YCHM	yeast chromosome marker
PCR	polymerase chain reaction

2. INTRODUCTION

A cell is the fundamental unit of life. It is indeed a huge aggregate of macromolecules (DNA, proteins, carbohydrates, lipids…) organized to carry out complex processes described as living. Molecular study of cell means the study of cell at a molecular level. It is concerned particularly with the structure and function of biological molecules. Understanding structure-function relationships of cell biomolecules is crucially important for the industrial exploitation of cells.

All organisms consist of cells, which fall into one of two types: prokaryotic (bacteria) or eukaryotic (yeasts and multicellular organisms). The useful physiological properties of yeast have led to their use in the field of biotechnology and they are also one of the most widely used model organisms for genetic and cell biology. Also in this work yeast was used as a model system.

Yeasts are easily grown unicellular eukaryotes. They are ubiquitous microorganisms, occuring in soil, fresh and marine water, animals, on plants and also in foods. The environment presents for yeast a source of nutrients and forms space for their growth and metabolism. On the other hand, yeast cells are continuously exposed to a myriad of changes in environmental conditions. These conditions determine the metabolic activity, growth and survival of yeasts. Basic knowledge of the effect of environmental factors on yeast is important for understanding the ecology and biodiversity of yeasts as well as for control the yeast physiology in order to enhance the exploitation of yeasts or to inhibit or stop their harmful and deleterious activity.

Environmental conditions that threaten the survival of a cell, or at least prevent it from performing optimally, are commonly referred to as cell stress. Environmental stress causes damage to many cellular components which stimulates the corresponding cell stress response. The molecular responses elicited by the cells dictate whether the organism adapts, survives, or, if injured beyond repair, undergoes death. An important aspect of each cellular response to environmental changes is the reorganization of genomic expression to the program required for growth in new environment. This altered genome expression results in alteration in the abundance of the corresponding gene products. Indeed, many of the changes in environmental stress response transcript levels correlate with changes in protein synthesis. Proteomic studies have identified proteins whose translation increases or decreases following starvation, osmotic shock, oxidative stress and heat shock. These transient changes in gene expression may help the cell to rapidly adjust the concentrations of the corresponding gene products to the levels required for growth at the new conditions. Functional analysis of the genome provides the tools for understanding the roles of gene products, their expression patterns, and how they interact to create a eukaryotic organism capable of complex processes like growth, cell division, and the response to extracellular signals. A number of different experimental approaches have been used to study gene expression in cells responding to different environments, and each method presents different features of stress responses.

The overproduction of some metabolites as part of cell stress response can be of interest to the biotechnology. For instance carotenogenic yeasts are well known producers of biotechnologically significant carotenoid pigments, namely astaxanthin, β-carotene, torulen, torularhodin and under stress conditions this carotenoid accumulation was reported to be increased. The use of this stressed biomass in feed industry could have positive effect not only in animal and fish feeds because of high content of physiologically active substances, but it could influence nutritional value and organoleptic properties of final products for human nutrition. Knowledge of molecular mechanism of the carotenoid production stimulation can then lead to improvement of such biotechnological process.

3. THEORY

3.1. YEAST CELL

A cell is the fundamental unit of life. It is indeed a huge aggregate of macromolecules organized to carry out complex processes described as living. All organisms consist of cells, which fall into one of two types: prokaryotic (bacteria) or eukaryotic. The eukaryotic cell evolved from a symbiotic community of prokaryotic cells. It is a complex, compartmentalized unit that differs from the prokaryotic cell by containing a nucleus and several other specialized structures called organelles. Eukaryotic cells are found in fungi, protozoa, algae, plants, and animals [1].

Yeasts are easily grown unicellular eukaryotes. They form crossing between bacteria and multicellular organisms. Thus, they are widely used as a model organism in modern cell biology research [2, 3].

3.1.1. Chemical composition of yeast cell

The chemical composition of yeast cell differs widely owing to differences in the yeast species and race and also in cultivation conditions and nutrient media. It is therefore not possible to set up a table that would contain generalized values of contents of major components in the yeast cell mass, but only the rough proportion in which a given component can be found in yeast [2, 3]

The yeast is comprised of the major elemental building blocks of macromolecules together with the bulk inorganic ions and trace elements which play a variety of structural and functional roles in the yeast cell [2].

Most elements do not exist naturally in pure, uncombined form but are bound together as molecules and compounds [1]. Organic compounds constitute the most important molecules in the structure and function of cells. Many of them are macromolecules (see *Table 1*) [1, 2].

Table 1: Macromolecular constituents of yeast cells [2]

Class of macromolecule	Function of macromolecule
Proteins	structural (actin, tubulin, histones, membrane proteins); ribosomal and functional (enzymes)
Glycoproteins	structural (mannoproteins); functional (invertase)
Polysaccharides	structural (cell wall glucan, mannan, chitin), storage (glycogen, trehalose)
Lipids	structural phospholipids (free sterols in membranes); storage lipids (sterol esters and triglycerides); functional lipids (phosphoglyceride derivatives in signal transduction) and free faty acids (in growth and metabolic processes)
Nucleic acids	DNA (approx. 80% is nuclear genomic DNA, 10 - 20% is mitochondrial genomic DNA and 1 - 5% is extrachromosomal); RNA (approx. 80% is rRNA and 5% is mRNA in cytoplasm)

Inorganic cell components are presented by inorganic ions (ash) and water. In brewer's and baker's yeasts ash constitutes 8 to 9 % of dry weight. The largest proportion of yeast ash is formed by phosphorus (P_2O_5, 35 to 65 %). The second most abundant element in yeast is potassium [2, 3].

Water makes up to 85 % of the cell mass and exists in both bound and free forms. Bound water ensures the necessary swelling properties of functional cell structures; for instance hydration of polysaccharides endows the cell wall and the plasmalemma with necessary elasticity and distensibility, hydration of proteins in the cytoplasm makes possible vital reactions, and in the superficial layers of cytoplasm water is responsible for selective permeability. Free water serves mainly as a transport vehicle during metabolism; it mediates the separation and apportioning of metabolic intermediates and drains off surplus heat [1, 3].

3.1.2. Yeast morphology

Yeast cells exhibit great diversity with respect to cell size, shape and colour.

The dimensions of the yeast cell are below the visibility limit of the naked eye and therefore can only be observed under a microscope. The size of most yeast cells falls within the range of 1 – 300 μm [3].

With regard to cell shape, yeasts have diverse shapes that are usually characteristic for certain species or genera within the variability limits. The cell shape changes under the influence of external conditions and is closely associated with the actual function of the cell. The basic shapes of yeast cells are taken to be ellipsoidal/ovoid, cylindrical with hemi-spherical ends, apiculate/lemon-shaped, ogival, flask-shaped, triangular, curved, filamentous, stalked, spherical or elongated (*Figure 1*) [2, 3].

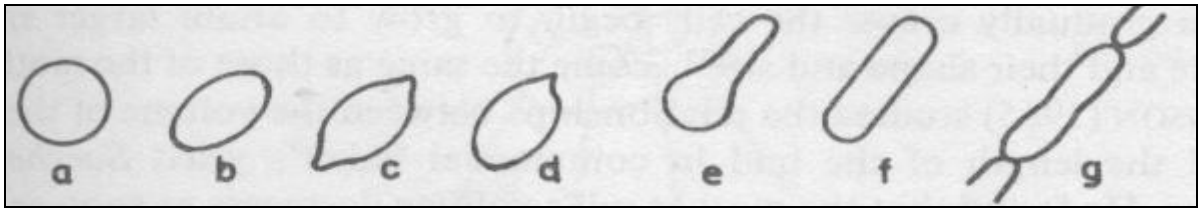

Figure 1: Yeast cell shapes [3]
(a - spherical, b - ellipsoidal, c - lemon-shaped,
d - ogival, e - bottle-shaped, f - elongated, g - filamentous)

Several yeasts are pigmented and the various colours may be visualized in surface-grown colonies: cream (*S. cerevisiae*), white, black (*Aureobasidium pullulans*), pink (*Phaffia spp.*), red (*Rhodotorula spp.*) … [2].

3.1.3. Yeast cell structure and function

In an idealized yeast cell (*Figure 2*), the following ultrastructural features can be observed: cell wall; periplasm; plasma membrane; invagination; bud scar; cytosole; nucleus; mitochondrion; ER, endoplasmic reticulum; Golgi apparatus; secretory vesicles; vacuole; peroxisome [4].

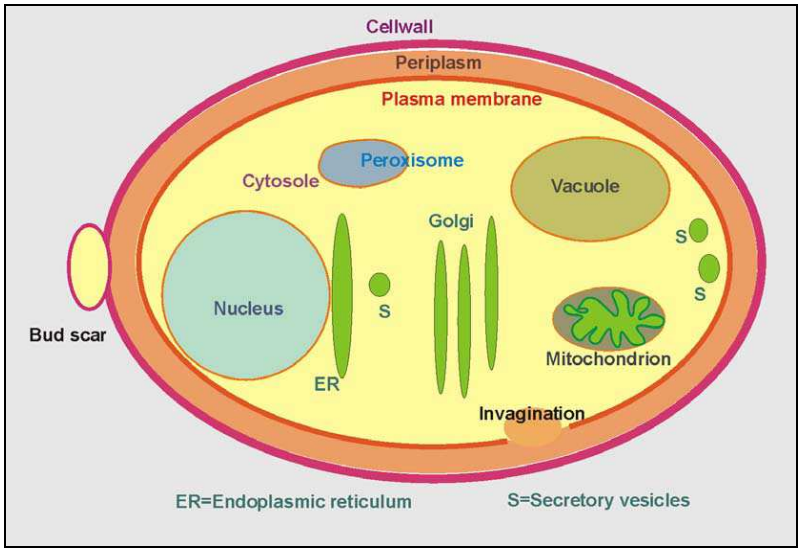

Figure 2: Scheme of organelles and compartments in a yeast cell [4]

Understanding structure-function relationships of yeast organelles and macromolecular structural organization is crucially important for the industrial exploitation of yeasts [2].

3.1.3.1. The cell envelope

The yeast cell envelope consists of the plasma membrane, the periplasmic space and the cell wall and, in certain yeasts, the capsule and other extracellular features. In *S. cerevisiae*, the cell envelope takes about 15% of the total cell volume and plays a major role in controlling the osmotic and permeabilty properties of the cell [2, 4].

The plasma membrane is about 7 nm thick, with some invaginations into the cytosole. It is a lipid bilayer interspersed with globular proteins. The lipid components comprise mainly phospholipids and sterols. Yeast membrane proteins include the following catagories: (I) cytoskeleton anchors; (II) enzymes for cell wall synthesis; (III) proteins for transmembrane signal transduction; (IV) proteins for solute transport (permeases, channels, ATPases) and (V) transport proteins. The primary functions of yeast plasma membranes are to provide selective permeability, i.e. to dictate what enters and what leaves the cytoplasm. Most important is the role of membrane proteins in regulating yeast nutrition, such as uptake of carbohydrates, nitrogenous compounds or ions, and the extrusion of molecules hazardous to the cell. Other important aspects include exo- and endocytosis of cargo molecules, stress responses, and sporulation [2, 4]

The yeast periplasm is a thin (35-45 A), cell wall associated region external to the plasma membrane and internal to the cell wall. It mainly contains secreted proteins (mannoproteins) that are unable to permeate the cell wall, but fulfill essential functions in hydrolysing substrates that do not cross the plasma membrane (invertase converts sucrose into glucose and fructose; acid phosphatase catalyzes the liberation of free phosphate from organic compounds) [2, 4].

The wall of yeast cell (*Figure 3*) is a remarkably thick (100 to 200 nm) structure comprising 15 to 25% of the total dry mass of the cell. Major structural constituents of yeast cell walls are polysaccharides (80-90%, mainly glucans and mannans, with a minor percentage of chitin). Other components of the cell wall are variable quantities of proteins, lipids, and inorganic phosphate. The yeast cell wall is a multifunctional organelle involved in cell protection, shape maintenance, cellular interactions, reception, attachment and specialized enzymatic activities [2, 4].

Bud scars are chitin-rich, ring-shaped, convex protrusions which remain on the mother cells of budding yeasts after birth of daughter cells. The concave indentations remaining on the surface of the daughter cell after budding are called birth scars [2, 4].

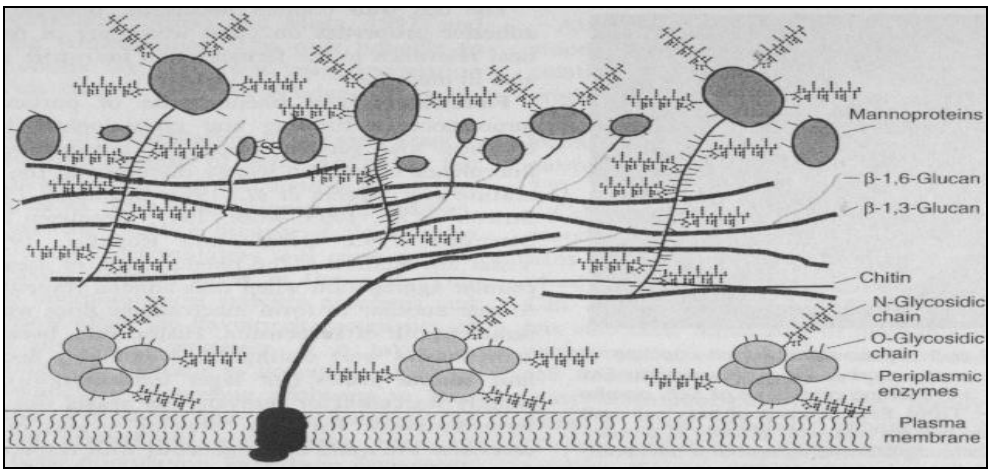

Figure 3: Composition and structure of the cell wall of *S. cerevisiae* [2]

3.1.3.2. The cytoplasm and cytoskeleton

The yeast cytoplasm is an acidic colloidal fluid containing ions and low or intermediate molecular weight organic compounds and soluble macromolecules (e.g. proteins, glycogen). The cytosolic enzymes of yeast include those of the glycolytic pathway, the fatty acid synthase complex and some enzymes for protein biosynthesis. The yeast cytoplasm contains several categories of microbodies: (I) freely-suspended yeast ribosomes; (II) lipid particles, which function as storage vesicles for yeast membrane biosynthesis; (III) peroxisomes; (IV) glyoxysomes and (V) proteasomes [2, 4].

The cytoskeletal network guaranteeing internal stability to the cell and providing structural organization comprises microtubules and microfilaments [2].

3.1.3.3. The nucleus

The yeast nucleus is a round-lobate organelle of around 1.5 μm diameter, separated from the cytosol by a double membrane containing pores between 50 to 100 nm in diameter. The nucleus contains a matrix called the nucleoplasm and a granular mass, the nucleolus. The nucleus is represented by the genomic DNA which together with histones and non-histones is organized into chromatin. Yeast chromosomes are formed and replicated during mitosis (or meiosis) but behave virtually invisible by microscopic techniques. However, pulsed field gel electrophoresis (PFGE) techniques provide convenient tools for chromosome separation and karyotyping. The nucleus ultimately governs and regulates all cell activities [1, 4].

In addition to the genomic material, yeast nuclei contain the machineries for DNA replication, DNA repair, transcription and RNA processing together with the necessary substrates and regulatory factors and the resulting (precursor) products, as well as a proportion of the yeast proteasomes [4].

Furthermore, several *non-chromosomal genetic elements* may be present in the yeast nucleus (2 μm DNA; killer plasmids: dsRNA and linear DNA) [2, 4].

3.1.3.4. The secretory system

The nucleus governs all cell activities through a structural and chemical network. Initially, a segment of the genetic code of DNA is copied into RNA and passed out through the nuclear pores directly to the ribosomes on the endoplasmic reticulum. Here, specific proteins are synthesized from the RNA code and deposited in the lumen (space) of the endoplasmic reticulum. After being transported to the Golgi apparatus, the protein products are chemically modified and packaged into vesicles that can be used by the cell in a variety of ways. Some of the vesicles contain enzymes to digest food inside the cell; other vesicles are secreted to digest materials outside the cell, and yet others are important in the enlargement and repair of the cell wall and membrane (lysosomes, vacuoles…) [1].

3.1.3.5. Mitochondria

Mitochondria are round to elongate particles scattered throughout the cytoplasm. A single mitochondrion consists of a smooth, continuous outer membrane that forms the external contour, and an inner, folded mebrane nestled neatly within the outer membrane. The folds on the inner membrane, called cristae, may be tubular, like fingers, or folded into shelflike bands. The cristae membranes hold the enzymes and electron carriers of aerobic respiration. This is an oxygen-using process that extracts chemical energy contained in nutrient molecules and stores it in the form of high-energy molecules, or ATP. The spaces around the cristae are filled with a chemically complex fluid called the matrix, which holds ribosomes, DNA, and the pool of enzymes and other compounds involved in the metabolic cycle. Mitochondria are unique among organelles in that they divide independently of the cell, contain circular strands of DNA, and have prokaryotic-sized 70S ribosomes [1, 2].

3.1.4. Yeast growth and metabolism

When microbes are provided with nutrients and the required environmental factors, they become metabolically active and grow. Growth takes place on two levels. On one level, a cell synthesizes new cell components and increases its size; on the other level, the number of cells in the population increases. This capacity for multiplication, increasing the size of the population by cell division, has tremendous importance in microbial control, infectious disease, and biotechnology [1].

A growth curve is a graphic representation of a closed population over time. It shows that the culture passes through several phases. The initial flat period of the curve is called the lag phase, followed by the exponential growth phase, in which viable cells increase in logarithmic progression. Adverse environmental conditions combine to inhibit the growth rate, causing a plateau, or stationary growth phase. In the death phase, nutrient depletion and waste buildup cause increased cell death [1].

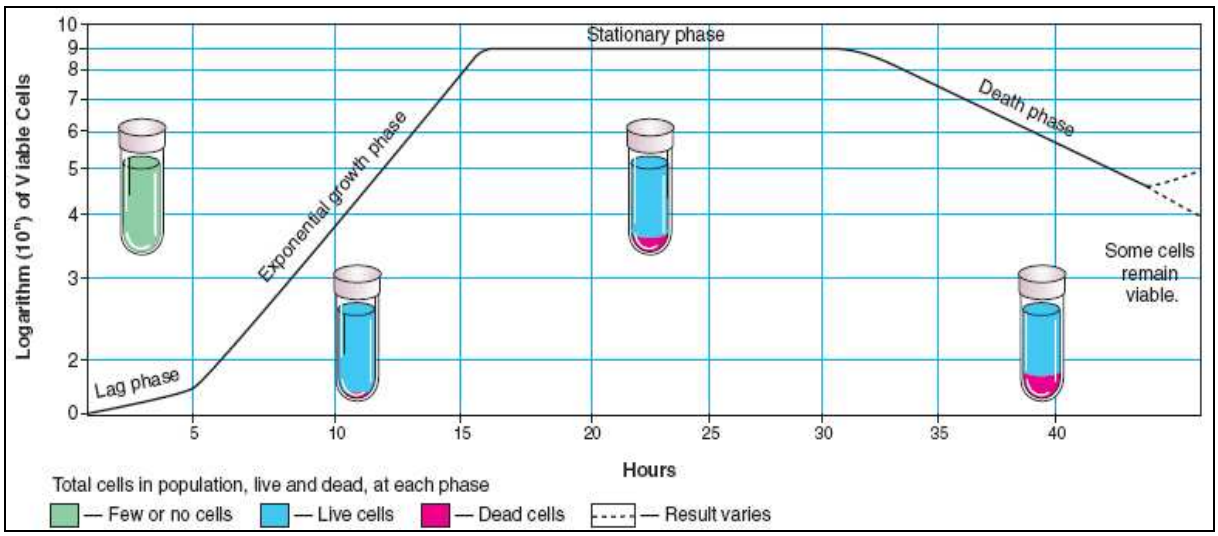

Figure 4: The growth curve [1]

Metabolism is the sum of cellular chemical and physical activities. It involves chemical changes to reactants and the release of products using well-established pathways. Metabolism is a complementary process consisting of anabolism (synthetic reactions that convert small molecules into large molecules) and catabolism (in which large molecules are degraded). Together, they generate thousands of intermediate molecular states, called metabolites, which are regulated at many levels. Knowledge of such regulation in yeasts is crucial for exploitation of yeast cell physiology in biotechnology [1].

Secondary metabolism (also called special metabolism) is a term for pathways and small molecule products of metabolism that are not absolutely required for the survival of the organism. Examples of the products include antibiotics and pigments. The induction of secondary metabolism is linked to particular environmental conditions or developmental stages. For example, when grown in a nutrient-rich medium, most bacteria employ almost solely basic metabolism in order to grow and reproduce. However, when nutrients are depleted, they start producing an array of secondary metabolites in order to promote survival [5].

3.1.5. Cytological methods

3.1.5.1. Microscopy

Since microorganisms are invisible to the unaided eye, the essential tool in microbiology is the microscope. Various types of microscopes are available for use in the microbiological laboratory. The microscopes have varied applications and modifications that contribute to their usefulness [1].

The bright-field microscope is the most widely used type of light microscope. It is a multipurpose instrument that can be used for both live, unstained material and preserved, stained material. At 1000-fold magnification, it may be possible to see the yeast vacuole and cytosolic inclusion bodies. By phase-contrast microscopy, together with appropriate staining techniques, several cellular structures can be distinguished. Fluorochromic dyes can be used with fluoresecence microscopy to highlight features within the cells as well as on the cell surface. The range of cellular features visualized is greatly increased, when monospecific antibodies raised against structural proteins are coupled to fluorescent dyes, such as fluorescein isothiocyanate or rhodamine B [1, 4].

Organelle ultrastructure and macromolecular architecture can only be obtained with the aid of electron microscopy, which in scanning procedures is useful for studying cell topology, while ultrathin sections are essential in transmission electron microscopy to visualize intracellular fine structure. Atomic force microscopy can be applied to uncoated, unfixed cells of imaging the cell surfaces of different yeast strains or of cells under different growth conditions [4].

3.1.5.2. Flow cytometry

Flow cytometry is a powerful technique for the analysis of multiple parameters of individual cells within heterogeneous populations. The properties measured include a particle's relative size, relative granularity or internal complexity and relative fluorescence intensity [6].

In the flow cytometer, particles are carried to the laser intercept in a fluid stream. Only one cell should move through the laser beam at a given moment. Any suspended particle or cell from 0.2–150 micrometers in size is suitable for analysis. When cell passes through the laser intercept, it scatters laser light. Any fluorescent molecules present on the particle fluoresce. The scattered and fluorescent light is collected by appropriately positioned lenses. A combination of beam splitters and filters steers the scattered and fluorescent light to the appropriate detectors. The detectors produce electronic signals proportional to the optical signals striking them [6].

Cell populations can be displayed in several different formats (*Figure 5*).

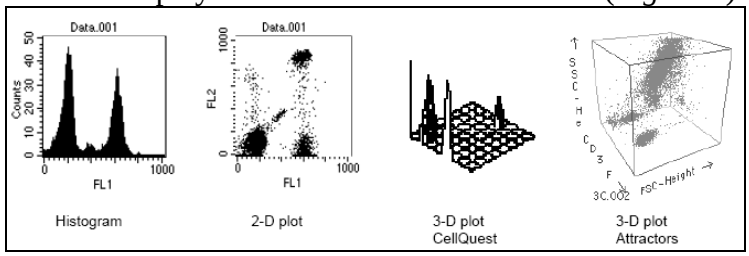

Figure 5: Graphic representations of flow cytometric data [6]

A specialized type of flow cytometry is fluorescence-activated cell sorting (FACS). It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, based upon the specific light scattering and fluorescent characteristics of each cell [6].

3.1.5.3. Biomass evaluation

The determination of the number of cells or concentration of cells in a sample plays numerous and important roles in microbiological characterization and experimentation. Classical methods for the determination of total biomass are based on either cell weight or cell enumeration. Various direct and indirect methods for the estimation of biomass have been developed using physical and biochemical techniques [1].

The traditional direct determination of biomass is by measurement of dry weight. Different methods are used for laboratory and industrial determination of yeast dry weight, e.g. differential weighing before and after drying and a variety of indirect methods such as centrifugation, turbidimetry, filtration, viscometry, etc. The contents of various cell components are usually referred to dry weight in order to facilitate comparison of different analyses [3].

The most accurate and sensitive method is direct counting by electronic counting ("Coulter" counter), flow cytometry or microscopic enumeration. Cell count can also be determined indirectly by turbidimetry [1].

3.1.5.4. Yeast cell disruption and fractionation

Disruption of yeast cell

To investigate intracellular components, these components need to be first released from the cell. Effective, well characterized methods of disruption are therefore required [7, 8].

Cell disruption can be achieved by both mechanical and non-mechanical methods:

- mechanical methods: the French press, shaking with glass beads and sonication (laboratory-scale methods) and bead milling, high-pressure homogenization, and microfluidization (process scale methods) [7, 8],
- non-mechanical methods: physical (decompression, osmotic shock and thermolysis), chemical (antibiotics, chelating agents, chaotropes, detergents, solvents and hydroxide and hypochlorite) and enzymatic (lytic enzymes, autolysis and cloned-phage lysis) [7, 8].

The various methods of disruption possess different modes of action. Those properties could be combined to give higher release of the product and make the process more effective. There are two approaches for modeling a combined method: (1) combination of chemical/physical/enzymatic methods and (2) pretreatment with a chemical/physical /enzymatic method followed by mechanical disruption [7, 8].

General problems associated with cell disruption include the liberation of DNA, heat generation and autolysis. Liberation of DNA can increase the viscosity of the suspension, which affect further processing. A nucleic acid precipitation step or the addition of DNase can help to prevent this problem. If heat is generated then proteins can denaturate unless appropriate cooling is implemented. Products released from eukaryotic cells are often subject to degradation by hydrolytic enzymes (proteases, lipases, etc.) liberated from disrupted lysosomes. This damage can be reduced by the addition of enzyme inhibitors, cooling the cell extract and rapid processing [8].

Cellular properties such as size and wall structure affect the ease of cellular disruption. Methods will vary depending on the type of cell and its particular cell wall structure. The method selected for large scale cell disruption will depend on susceptibility of cells to disruption, product stability, ease of extraction from cell debris, speed of method and cost of method. The choice of the disruption method has to be made empirically, at the same time taking into consideration the subsequent processing steps [7, 8].

The disruption process is often quantified by monitoring changes in absorbance, particle size, total protein concentration or the activity of a specific intracellular enzyme released into the disrupted suspension [8].

By careful optimization of physical disruption techniques, detergent-buffer solutions and density gradient methods, procedures have been developed to enable separation of subcellular structures or classes of compounds. A combination of tools and steps enable intact nuclei, mitochondria and other organelles to be isolated for study or protein solubilization [9].

DNA isolation

Many different methods and technologies are available for the isolation of genomic DNA. In general, all methods involve disruption and lysis of the starting material followed by the removal of proteins and other contaminants and finally recovery of the DNA. Removal of proteins is typically achieved by digestion with proteinase K, followed by salting-out, organic extraction, or binding of the DNA to a solid-phase support (either anion-exchange or silica technology). DNA is usually recovered by precipitation using ethanol or isopropanol. The choice of a method depends on many factors: the required quantity and molecular weight of the DNA, the purity required for downstream applications, and the time and expense [10].

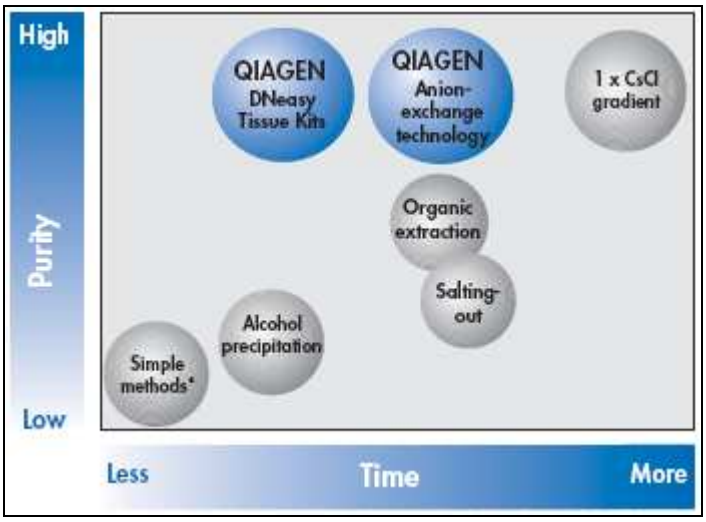

Figure 6: A comparison of common methods of genomic DNA isolation [10]

Protein extraction and purification

Due to the great diversity of protein sample, it is difficult to establish one general method for their extraction and purification. The optimal procedure must be determined empirically for each sample type. Ideally, the process will result in the complete solubilisation of the proteins in the sample [11].

The proteins are extracted with a so-called “lysis buffer”. The components of the lysis buffer have to get proteins into solution, prevent different oxidation steps and protein aggregates and deactivate proteases. A standard lysis buffer contains [11]:

- urea, thiourea, SDS or TCA (to get and keep hydrophobic proteins in solution and avoid protein–protein interactions, but they denaturate proteins);
- zwitterionic or non-ionic detergent like CHAPS, Triton X-100 or NP-40 (to increase the solubility of hydrophobic proteins);
- reducing agents like DTT or DTE (to prevent different oxidation steps);
- protease inhibitors.

After this extraction process, proteins will be in the solvent, and need to be separated from non-protein part of the mixture (cell membranes, DNA etc.) by centrifugation. Finally proteins have to be separated from each other. The purification methods exploit differences in protein size (size exclusion chromatography, gel electrophoresis ...), physico-chemical properties (ion exchange chromatography) and binding affinity (liquid chromatography) [12].

3.1.6. Yeast as a model system

Yeast species provide excellent models for fundamental biological research. In the laboratory, two species are commonly employed as models for biological research: the budding or brewer's yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* (*Figure 7*). They have substantial similarities that make them powerful as research tools, and also striking biological differences that make them complementary experimental models. Both yeasts are harmless, tractable genetic systems, easily manipulated in the laboratory using superb molecular tools. Each provides unique tools for understanding environmental effects on cellular systems [13].

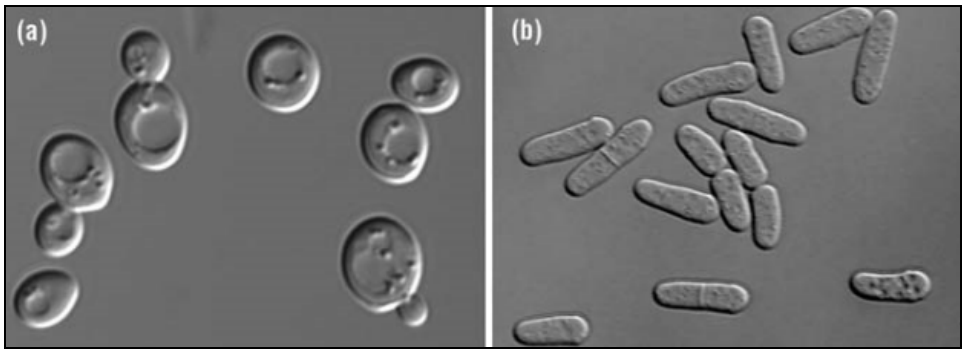

Figure 7: The budding yeast *S. cerevisiae* (a) and the fission yeast *S. pombe* (b)

Saccharomyces cerevisiae

A model organism and the first eukaryote to have its genome sequenced *Saccharomyces cerevisiae* had been at the forefront of eukaryotic cellular and molecular biology for more than 50 years [14]. With its basic genetics, biochemistry, and cellular biology established many decades ago, *S. cerevisiae's* autonomously replicating plasmid, whole cell transformation system and the ability rapidly to form discrete colonies on simple defined media ensured that it remained at the forefront of developments during the recombinant DNA revolution. Within a very few years, this extremely tractable model organism rapidly yielded up a whole series of molecular secrets on a global scale: each of its genes was systematically deleted in search of phenotypes, technology to allow its global mRNA profiles to be identified was developed and all possible protein–protein interactions were examined. *S. cerevisiae* became a central player in the development of an entirely new approach to biological research: systems biology. This newly emerging field uses a cross-disciplinary approach to develop working computer models of how molecules interact to generate biological phenomena. This simple eukaryote is placed to address many questions of fundamental biological importance and has become a central player in postgenomic research [13-15].

Schizosaccharomyces pombe

Sz. pombe is a linear, rod shaped cell that grows at its ends and divides by medial fission, producing two essentially identical daughter cells. This fission yeast is a popular model eukaryote that has become particularly useful in studies of cell cycle and chromosome dynamics. With a haploid genome size of just 13.8 Mb, distributed amongst 4824 ORFs (compared to over 5500 for *S. cerevisiae*), *Sz. pombe* has the smallest sequenced eukaryotic genome. Like budding yeast, fission yeast is genetically tractable, and lends itself to easy molecular manipulation. Most tools available in *S. cerevisiae* are available in *Sz. pombe* versions that accommodate the distinct biology of the fission yeast, and similar genetic strategies are available for both systems. General handling of the organism, microbial culture and overall purification of nucleic acids and proteins are similar to budding yeast [13, 16].

3.2. YEASTS AND ENVIRONMENT

Yeasts are ubiquitous microorganisms. They occur in soil, fresh and marine water, on plants, are commonly associated with animals and are also found frequently in man-made habitats such as foods [17].

The environment presents for yeasts a source of nutrients and forms space for their growth and metabolism. On the other hand, yeast cells are continuously exposed to a myriad of changes in environmental conditions (reffered to as environmental stress). These conditions determine the metabolic activity, growth and survival of yeasts. Basic knowledge of the effect of environmental factors on yeast is important for understanding the ecology and biodiversity of yeasts as well as to control the environmental factors in order to enhance the exploitation of yeasts or to inhibit or stop their harmful and deleterious activity [17].

3.2.1. Yeast requirements

Cellular organisms require specific internal conditions for optimal growth and function. The state of this internal milieu is strongly influenced by chemical, physical and biological factors in the growth environment. Understanding yeast requirements is important for successfull cultivation of yeast in the laboratory but also for optimalization of industrial fermentation process [2].

3.2.1.1. Yeast nutritional requirements

Elemental composition of yeast cell gives a broad indication as to the nutritional reguirements of the yeast cell. Yeasts acquire essential elements from their growth environment from simple food sources which need to be available at the macronutrient level (approx. 10^{-3} M) in the case of C, H, O, N, P, K, Mg and S or at the micronutrient level (approx. 10^{-6} M) in the case of trace elements. Yeasts are chemoorganotrophs as they use organic compounds as a source of carbon and energy. *Table 2* summarizes the main nutritional requirements of yeasts [2].

Table 2: Elemental requirements of yeasts [2]

Element	Common sources	Cellular functions
Carbon	sugars	major structural element of yeast cells in combinatin with hydrogen, oxygen and nitrogen; energy
Hydrogen	protons from acidic environments	transmembrane proton-motive force vital for yeast nutrition; intracellular acidic pH (around 5 – 6) necessary for yeast metabolism
Oxygen	air, O_2	substrate for respiratory and other mixed-function oxidative enzymes; essential for ergosterol and unsaturated fatty acid synthesis
Nitrogen	NH_4^+ salts, urea, amino acids	structurally and functionally as organic amino nitrogen in proteins and enzymes
Phosphorus	phosphates	energy transduction; nucleic acid and membrane structure
Potassium	K^+ salts	ionic balance; enzyme activity
Magnesium	Mg^{2+} salts	enzyme activity; cell and organelle structure
Sulphur	sulphates, methionine	sulphydryl amino acids and vitamins
Calcium	Ca^{2+} salts	possible second messenger in signal transduction
Copper	cupric salts	redox pigments
Iron	ferric salts	haem-proteins; cytochromes
Manganese	Mn^{2+} salts	enzyme activity
Zinc	Zn^{2+} salts	enzyme activity
Nickel	Ni^{2+} salts	urease activity
Molybdenum	Na_2MoO_4	nitrate metabolism, vitamin B12

3.2.1.2. Physical requirements for yeast growth

Microbes are exposed to a wide variety of environmental factors in addition to nutrients, such as heat, cold, gases, acid, radiation, osmotic and hydrostatic pressures, and even other microbes [1].

Temperature

Temperature is one of the most important physical parameters which influence yeast growth. All microorganisms exhibit characteristic minimum, optimum and maximum growth temperatures. According to thermal domains for growth, yeasts can be grouped as psychrophiles, mesophiles and thermophiles. Most laboratory and industrial yeasts are mesophillic; they generally grow best between 20-30°C [2].

Water potential

All life processes is taking place in the water. All living organisms need water in high concentrations for growth and metabolism. To quantitate the availability of water in the presence of dissolved solutes, the term water potential is used. As is the case with temperature, yeasts have cardinal water potentials for growth (minimal, optimal and maximal). Although most microbes exist under hypotonic or isotonic conditions, a few, called halophiles, live in habitats with a high solute concentration. Obligate halophiles grow optimally in solutions of 25% NaCl but require at least 9% NaCl (combined with other salts). Facultative halophiles are remarkably resistant to salt, even though they do not normally reside in high-salt environments. The term to describe microbes that withstand and grow at high osmotic pressures is osmophile [1, 2].

Media pH

Most yeasts grow very well between pH 4.5 and 6.5, but nearly all species are able to grow, albeit to a lesser extent, in more acidic or alkaline media [2].

A few microorganisms live at pH extremes. Obligate acidophiles grow in acid environment at a pH of 0 to 2. Alkalinophiles live in hot pools and soils that contain high levels of basic minerals (up to pH 10.0) [1].

Oxygen

With respect to oxygen requirements, several general categories are recognized. An aerobe can use oxygen in its metabolism. An organism that cannot grow without oxygen is an obligate aerobe. A facultative anaerobe is an aerobe that is capable of growth in the absence of oxygen. A microaerophile does not grow at normal atmospheric tensions of oxygen but requires a small amount of it in metabolism. An anaerobe lacks the metabolic enzyme systems for using oxygen in respiration. Because strict, or obligate, anaerobes also lack the enzymes for processing toxic oxygen, they cannot tolerate any free oxygen in the immediate environment and will die if exposed to it. Aerotolerant anaerobes do not utilize oxygen but can survive and grow to a limited extent in its presence [1].

Miscellaneous environmental factors

Various forms of electromagnetic radiation (ultraviolet, infrared, visible light) stream constantly onto the earth from the sun. Yeasts tend to be damaged by the toxic oxygen products produced by contact with light. Some microbial species produce carotenoid pigments to protect against the damaging effects of light by absorbing and dismantling toxic oxygen. Other types of radiation that can damage microbes are ultraviolet and ionizing rays (X rays and cosmic rays) [1].

3.2.1.3. Yeast cultivation

Cultivation techniques for growing microbes out of their natural habitats and in pure form in the laboratory were developed. These techniques enabled the close examination of a microbe and its morphology, physiology, and genetics [1].

For successful cultivation, each microorganism had to be provided with all of its required nutrients in an artificial medium [1, 2].

Nutritional requirements of microbes vary from a few very simple inorganic compounds to a complex list of specific inorganic and organic compounds. This tremendous diversity is evident in the types of media that can be prepared. At least 500 different types of media are used in culturing and identifying microorganisms. Culture media are contained in test tubes, flasks, or Petri plates and they are inoculated by such tools as loops, needles, pipettes, and swabs. Media are extremely varied in nutrient content and consistency and can be specially formulated for a particular purpose. For an experiment to be properly controlled, sterile technique is necessary. This means that the inoculation must start with a sterile medium and inoculating tools with sterile tips must be used. Measures must be taken to prevent introduction of nonsterile materials such as room air and fingers directly into the culture.

Media can be classified on three primary levels: (1) physical form, (2) chemical composition, and (3) functional type (See *Figure 8*) [1, 2].

Physical State (Medium's Normal Consistency)	Chemical Composition (Type of Chemicals Medium Contains)	Functional Type (Purpose of Medium)*
1. Liquid 2. Semisolid 3. Solid (can be converted to liquid) 4. Solid (cannot be liquefied)	1. Synthetic (chemically defined) 2. Nonsynthetic (not chemically defined)	1. General purpose 2. Enriched 3. Selective 4. Differential 5. Anaerobic growth 6. Specimen transport 7. Assay 8. Enumeration

Figure 8: Media classification [1]

Once a container of medium has been inoculated, it is incubated. An incubator can be used to adjust the proper growth conditions of a sample. Setting the optimum temperature and gas content promotes multiplication of the microbes over a period of hours, days, and even weeks. Cultivation can take place in different ways [1]:

- batch cultivation - is the most common laboratory growth environment. The system of batch culturing is closed, meaning that nutrients and space are finite and there is no mechanism for the removal of waste products.
- fed-batch cultivation – semi-continuous process
- chemostat/continuous culture system - can admit a steady stream of new nutrients and siphon off used media and old cells, thereby stabilizing the growth rate and cell number. The chemostat is very similar to the industrial fermenters, it has the advantage of maintaining the culture in a biochemically active state and preventing it from entering the death phase [2].

Laboratories maintain a line of stock cultures (frozen or freeze-dried) that represent "living catalogues" for study and experimentation [1].

3.2.2. Environmental stress

Single-celled organisms living freely in nature, such as yeasts, face large variations in their natural environment. Environmental conditions that threaten the survival of a cell, or at least prevent it from performing optimally, are commonly referred to as *cell stress*. These environmental changes may be of a physical or chemical nature: temperature, radiation, concentrations of solutes and water, presence of certain ions, toxic chemical agents, pH and nutrient availability. In nature, yeast cells often have to cope with fluctuations in more than one such growth parameter simultaneously [15].

In industry, yeast stress has several very important practical implications. In brewing, for example, if yeast is nutrient-starved during extended periods of storage, certain cell surface properties such as flocculation capability are deleteriously affected [2].

3.2.2.1. Thermal stress

High temperature stress (heat shock or hyperthermia)/low temperature stress (cold shock) is caused by exposure of cells to temperature which is higher/lower than temperature optimal for their growth, i.e. supraoptimal/suboptimal temperature. The range of temperatures which can be defined as supraoptimal/suboptimal varies depending on normal growth and so on yeast species [18, 19].

Structural and morphological effects caused by temperature

When temperature is increased there is an increase in the rotational, vibrational, and translational kinetic energy of all cellular molecules. There are three direct consequences of increased kinetic energy [18]:

1. Increased membrane fluidity - which can lead to a disruption of membrane structure and function.
2. Increased metabolism (i.e. biochemical reactions).
3. Transitions - occur in numerous cellular components, such as proteins (protein denaturation), lipids (lipid phase changes), DNA (DNA melting), etc.

The protein denaturation is the most significant transition. The thermally denatured state of a protein has two important characteristics: inactivation of crucial cellular functions (e.g. loss of enzyme activity) and aggregation (due to exposure of hydrophobic residues). Both of these characteristics lead to cell damage [18].

Low temperature results in a phase transition of membrane fatty acids/sterols from fluid to “gel” states. Hence membrane integrity is compromised such that it leaks and yeast cells uniformly shrink [2].

Measurements of effect of temperature on cells

Numerous techniques exist for measuring protein denaturation. Any parameter sensitive to protein conformation can be used. These include fluorescence spectroscopy, electron paramagnetic resonance, spin labeling, circular dichroism, nuclear magnetic resonance and Raman spectroscopy. The general requirement is that the measured parameter has a different value in the denatured, unfolded state (YD) than in the native, folded state (YN). Then YDN = YD – YN gives the extent of denaturation [18].

An alternate approach is to measure a property of the transition itself. Temperature induced transitions, such as protein denaturation, are endothermic. Thus, heat is absorbed during the transition. Measurement of the extent of heat absorbed, the calorimetric enthalpy, is a measure of the extent of the transition. Differential scanning calorimetry is based on this principle [18, 20].

3.2.2.2. Oxidative stress

Oxidative stress originates from toxic levels of oxygen-derived reactive species (ROS) [15, 21].

Several compounds are used in yeast to generate oxidative stress. H_2O_2 is highly diffusible and relatively unreactive which make it likely to penetrate all cellular compartments and to react with any reactive molecule. Organic peroxide derivatives that have been used in yeast are tert-butyl hydroperoxide, cumene hydroperoxide, linolenic, and linoleic acid hydroperoxide. Their hydrophobicity makes them more likely to react with membrane components. In *S. cerevisiae*, toxic concentrations of peroxides are in the range of 0.05 mM for lipid hydroperoxides to up to 5 mM for H_2O_2, but can vary among yeast species. Redox cycling chemicals, such as menadione, paraquat and plumbagin are used to generate O_2^- in aerobically growing cells. Menadione is toxic in the hundred-micromolar range in *S. cerevisiae*. Other compounds, such as diamide and diethylmaleate, cause oxidative stress indirectly. These chemicals are toxic in the millimolar range [15, 21].

REACTIVE OXYGEN SPECIES (ROS)

A free radical is defined as a molecule with one or more unpaired electrons in an outermost valence shell. ROS include O_2-derived free radicals: superoxide anion radical (O_2-) and the hydroxyl radical (•OH), as well as nonradical derivates of O_2 (H_2O_2) [21-23].
According to the ROS origin, we distinguish:

1. Primary ROS
   - *the superoxide anion*, O_2-
     O_2- is mainly generated from the leakage of electrons from the mitochondrial respiratory chain as a normal consequence of aerobic respiration. It is also formed in microsomal metabolism and during the respiratory burst produced by phagocytes. The superoxide anion is not strongly reactive, but can react directly with some proteins.
   - *the hydrogen peroxide*, H_2O_2
     Hydrogen peroxide is produced during the detoxification of superoxide anion catalysed by superoxide dismutases, also in yeasts during the oxidation of fatty acids in the peroxisome, various oxidase reactions and also from the protein folding processes in the ER. H_2O_2 can readily cross most biological membranes. While it is relatively unreactive, it has deleterious effects through its conversion to the extremely reactive hydroxyl radical.
   - *the hydroxyl radical*, OH•
     OH• is formed via the Fenton and Haber-Weiss reactions involving the combined action of superoxide anion and H_2O_2, catalysed by transition metal ions such as Fe^{2+}. The hydroxyl radical is the most highly reactive ROS. This high reactivity limits its diffusion in biological systems, causing OH• to generate cellular damage as soon as it is formed. OH• can abstract hydrogen from a molecule leading to a new radical species. It can also react by addition on molecules containing aromatic rings such as purine and pyrimidine bases, or to metals forming metal-centered radicals. Lastly, OH• can transfer its unpaired electron to another molecule to create a new radical species. These chemical properties explain the wide range of reactions, often chain reactions, in which OH• is engaged.

2. Secondary ROS which are generated from the action of primary ROS (see next) [21, 22].

Structural and morphological effects caused by oxidative stress

Elevated nonphysiological concentrations of ROS can cause the progressive modification or degradation of cellular macromolecules, including DNA, proteins, lipids and carbohydrates [21, 22].

The peroxidation of lipids represents a primary consequence of cellular oxidative stress. Lipid peroxidation refers to the reaction of ROS with unsaturated fatty acids resulting in autocatalytic formation of lipid radicals and toxic lipid hydroperoxides. The oxidation of membrane phospholipids in the plasma membrane, as well as within internal organelle membranes such as the mitochondria, leads to biophysical changes that disrupt membrane and organelle function. Lipid derived peroxidation products and some of their by-products, malondialdehyde (MDA) and 4-hydroxynonenal, promptly react with other biological molecules, principally forming adducts with DNA bases and proteins [15, 21, 22, 24].

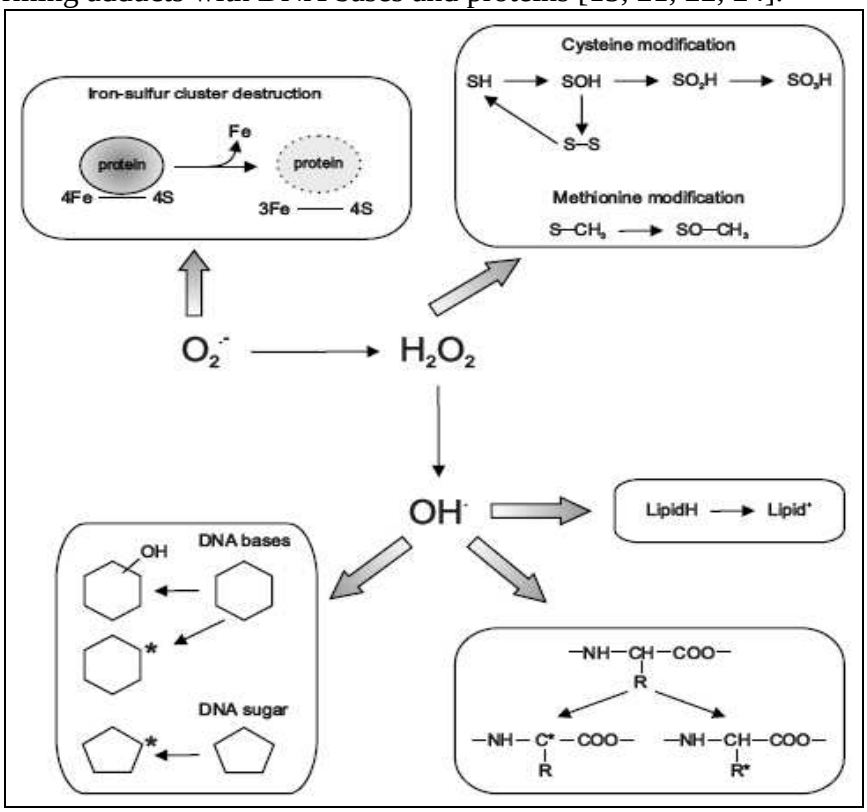

Figure 9: The reactivity of ROS towards biological molecules [15]

Free radical attendance can also lead to cross-linking and fragmentation of proteins leading to enhanced proteolysis. Hydroxyl radicals cause oxidation of amino acid residues, especially aromatic amino acid residues and cysteine. Incurred reactive protein hydroperoxides can decompose which lead to free radicals causing modification of amino acids on the protein backbone, fragmentation, cross-linking and unfolding. Hydroxylated derivatives are generated from aliphatic amino acids. Other protein modifications are generated by by-products of lipid peroxidation (4-HNE and MDA) and DNA oxidation through nucleophilic addition of side chains residue [21, 22, 24].

ROS can damage also nucleic acids and they have been implicated in mutagenesis and carcinogenesis. Only the hydroxyl radical and singlet oxygen are considered energetic enough to affect DNA directly, and H_2O_2 and superoxide anion cause damage to nucleic acids via the generation of the hydroxyl radical. Actually, any other radicals that are formed from lipid and protein peroxidation can induce DNA damage. Modifications of DNA by the OH• include DNA single and double strand breaks, direct base modifications, abasic site formation, and DNA cross-links. Half of the modifications induced by OH• occur on DNA bases [21, 22, 25].

Measurement of the ROS toxicity

Measurements of ROS attendance

The presence of ROS can be detected by dyes that fluoresce when oxidized (fluorochromes), such as dihydroethidium, which is moderately specific for superoxideanion, and dihydrorhodamine 123 and 2,7-dichlorodihydrofluoroscein diacetate, which are oxidised by a broader range of ROS [15, 22].

Measurement of cell sensitivity to ROS

The "challenge/survival assay" measures the ability of cells to survive an acute exposure to ROS. It consists of exposing a cell suspension to increasing concentrations of a ROS, and counting the number of remaining viable cells after a one or two hour's incubation period by their ability to form colonies on agar plates.

The "patch/plate sensitivity assay" measures the ability of cells to survive and grow under a chronic exposure to ROS. It consists of examining the growth of cells that have been inoculated on solid medium containing increasing concentrations of a ROS after a three-day period.

Growth inhibition is also monitored by recording the growth rate of cells grown in liquid medium in the presence of a ROS. More specific assays for monitoring growth inhibition/growth arrest rely on the measurement of the bud index and of the cellular DNA content by flow cytometry of asynchronous or synchronous cell populations [15].

Methods for the analysis of ROS cellular damage

Oxidative damage to proteins can be evaluated by the titration of carbonyl groups generated in some amino acid side chains during stress conditions. Detection of carbonyls in proteins takes advantage of their reactivity to dinitrophenyl hydrazine (DNPH). DNPH-modified proteins are detected by spectrophotometry or revealed by anti-DNPH antibodies. The cysteine reduced SH status of a specific protein can be monitored by changes of its electrophoretic mobility resulting from the differential alkylation of cysteine SH groups with high molecular weight alkylating agents [15, 26, 27].

Lipid peroxidation is detected by the measure of its by-products malondialdehyde by chromatographic methods or by the thiobarbituric acid reactive substance (TBARS) method combined with fluorometry [15, 27].

Mutagenicity of a ROS is monitored via genetic screens with reporter genes that act as genetic markers and their sequencing [15].

8-hydroxyguanine (8OHG) has become the most widely analysed chemical biomarker for oxidative DNA damage by a simple electrochemical detection method linked to HPLC. The comet assay measures single and double strand breaks by a single cell gel electrophoresis technique. A challenging future development of this sensitive method is the application of fluorescent in-situ hybridisation (FISH) to investigate the integrity of specific genes within the damaged DNA. Chromosomal DNA fragmentation can be detected by gel electrophoresis but also in situ by DNA staining methods such as terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), nick translation or in situ ligation. By TUNEL staining both single- and double-strand DNA breaks can be indicated, whereas in situ ligation specifically stained double-strand DNA breaks. DNA fragments of several hundred kilobases are too large to be detected by normal DNA agarose electrophoresis and therefore pulsed field gel electrophoresis of chromosomal DNA is used for [25, 28].

3.2.2.3. Water/osmotic and saline stress

Osmotic stress is caused by changes in the concentration of dissolved molecules in the medium, thereby altering the availability of water. Increasing osmolarity will lead to a decreased water activity and water potential and vice versa. Water always flows across a semi-permeable membrane towards the higher solute concentration, i. e. towards the lower water activity or higher osmolarity. Since biological membranes are more permeable to water than to most solutes, living cells are affected by changes in total solute concentration. Consequently, an increase in external osmolarity (hyperosmotic stress) will lead to water outflow from a cell, whereas a decrease in external osmolarity (hypoosmotic stress) will cause water inflow. Microorganisms keep a slighty higher internal osmotis pressure than that of the surrounding medium. This pressure difference is counteracted by cell wall resistance and is reffered to as cell turgor pressure [2, 15, 29].

In the laboratory, osmotic stress is often created by adding NaCl or sorbitol to the growth medium. Exposure of yeast cells to NaCl results not only in osmotic stress but it is also accompanied by Na^+ toxicity reffered to as saline stress [15, 29].

Structural and morphological effects caused by water stress

Alterations in water availability elicit changes in cell morphology and structure. A hyperosmotic shock causes rapid water outflow accompanied by cell shrinking. A hypo – osmotic shock increases the water concentration gradient and leads to rapid influx of water, cell swelling and hence increased turgor pressure. Within wide limits, the yeast cell wall prevents cell bursting. Osmotic stress also perturbs the plasma membrane at the level of its structure, permeability and mechanic properties [15, 29].

Na^+ toxicity

Na^+ ions are toxic to cells due to their ability to inhibit specific metabolic pathways, probably through inhibition of specific targets. This has been shown to be the case for the yeast Hal2 protein and certain RNA-processing enzymes [30].

Measurements of effect of osmotic stress on cells

Osmotic changes can be controlled very well experimentally by microscopic techniques [2, 29].

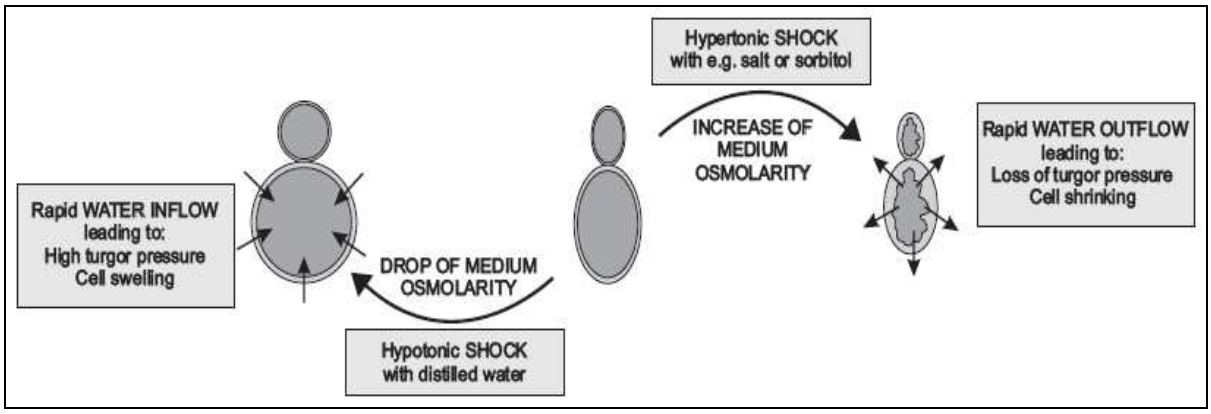

Figure 10: Effect of osmotic stress on yeast cell [15]

3.2.2.4. Nutritional stress – nutrient limitation/starvation

Starvation is a complex, albeit common, stress for microorganisms. The nutrients for which a cell can be starved include carbon and nitrogen, with other elements such as phosphate, sulfur, and metals being less commonly evaluated. Yeasts can use a wide variety of substances as nutrient sources. Decreasing availability of one substrate can, in many instances, be compensated by the utilisation of another [15, 31].

When a single essential nutrient becomes limiting and eventually absent, the cellular proliferative machinery is efficiently shut down and a survival program is launched. In the absence of any one of the essential nutrients, yeast cells enter a specific, non-proliferative state known as stationary phase, with the ultimate aim of surviving the starvation period. In the presence of a poor carbon source, starvation for nitrogen induces sporulation and in the presence of a good carbon source stimulates pseudohyphal growth [15, 31].

3.2.2.5. Other stresses

Radiation stress

Radiation stress means exposure of cells to unphysiological doses of ionizing radiotion, such as ultraviolet, gamma ray irradiation, near-infrared high-power laser light, diffuse daylight, etc. Ultraviolet (UV) and gamma ray irradiation are known to cause DNA damage in yeast cells. UV causes dimerization, nicks and lesions while penetrating γ-rays induce double-strand breaks, nicks and other damaging effects on yeast DNA. The main effects of ionizing radiation on yeast cells are indirect effects mediated by ROS (hydroxyl radicals generated by water radiolysis) [2].

Heavy-metal stress

Various metal ions (e. g. Cu, Cd) represent a severe chemical stress toward yeast cells due to their effects on enzyme inactivation and membrane damage. In addition, heavy metal excess may stimulate the formation of free radicals and reactive oxygen species [2, 32].

Chemical mutagenesis

Chemical mutations are caused by toxic chemicals that react with DNA and alter the chemical structure of the bases. The agents that mutate DNA are called mutagens. The most common mutagens are alkylating agents (methyl methane sulfonate and ethyl methane sulfonate) which alkylate DNA, proteins, and likely other cellular components [33, 34].

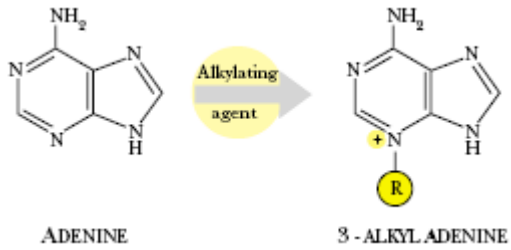

Figure 11: Base alteration by alkylating agents [34]

Environmental stress causes damage to many cellular components which stimulates the corresponding repair mechanisms, reffered to as environmental stress response. The molecular responses elicited by the cells dictate whether the organism adapts, survives, or, if injured beyond repair, undergoes death [4, 15].

3.3. THE ENVIRONMENTAL STRESS RESPONSE

3.3.1. Definition of environmental stress response

Unicellular organisms require specific internal conditions for optimal growth and function. Sudden changes in the external environment can perturb the internal milieu and disrupt normal processes. Therefore, cells must maintain their internal system despite fluctuations in the external surroundings. The mechanism that yeasts use to protect the internal system from the effects of environmental variation is reffered to as *the environmental stress response* (ESR). The ESR is of vital importance to all living organisms, ranging from simple prokaryotes to multicellular eukaryotes. Underlying basic mechanisms seem to possess the high degree of evolutionary conservation, especially within the eukaryotic kingdom [15].

The severity of a stress affects the profile of a response dramatically. At low doses the cells can adapt by activation of various defence functions. At higher doses, death of a proportion of cells in the population occurs, initially by apoptosis, at extreme doses by necrosis. The more severe a stress the longer it takes the cell to respond [15, 22].

ADAPTATION

The aim of the adaptive stress response is protection of cells from the detrimental effects of environmental stress and reparation of possible damage. Response, in this case, increases organism's tolerance to new environment. Tolerance to deleterious factors (e.g., low pH) refers to a microorganism's ability to survive a stress. This phenomenon is described as adaptive response, induced tolerance, habituation, acclimatization or stress hardening [15, 35].

Once cells have been challenged with a mild stress, they become more resistant to severe stress. Also exposure to one type of stress has been demonstrated to lead to tolerance to other types of stress as well (*cross-protection*) [15].

APOPTOSIS

Apoptosis represents a regulated form of cell death that requires the action of proteases and nucleases within an intact plasma membrane. Mitochondrial dysfunction, respiratory chain inhibition, loss of inner mitochondrial membrane potential and increased mitochondrial membrane permeability represent cardinal biochemical features of apoptosis. Morphological characteristics of apoptosis include DNA fragmentation, membrane blebbing, cell shrinkage and cellular decomposition into membrane-bound apoptotic bodies destined for phagocytosis. Apoptosis serves a critical function in the maintenance of cell homeostasis under physiological conditions, as a component of developmental programs [21].

NECROSIS

In necrosis, an extensive cell lysis results from acute, accidental or nonphysiological injury. This type of cell death is associated with gross membrane damage and leakage of cell constituents into the extracellular space [21].

3.3.2. Mechanism of adaptative environmental stress response

Cells respond to sudden stress in different phases:

1. *The primary phase* is represented by immediate cellular changes occuring as a direct consequence of stress exposure (see chapter 3.2.2.) [15].
2. *The secondary phase – the stress-responsive phase* is characterised by sensing, signalling and adjustment of gene and protein expression.

   Immediate cellular changes are detected by specific sensory systems, which activate specialized signaling pathways to transmit a cellular signal to the nucleus. The upstream signaling factors activate multiple downstream protein kinases, each of which in turn likely activates a host of proteins that mediate cellular responses such as cell cycle arrest, translation inhibition, gene and protein expression changes and enzymatic alterations [15].
3. *Adaptative phase*

   Each cellular response is precisely orchestrated to result in a unique cellular program that will ensure survival of the cell in the new environment. By other words, integration of all stress-induced cellular activities leads to resumption of growth and proliferation, i.e. adaptation to new environment [15].

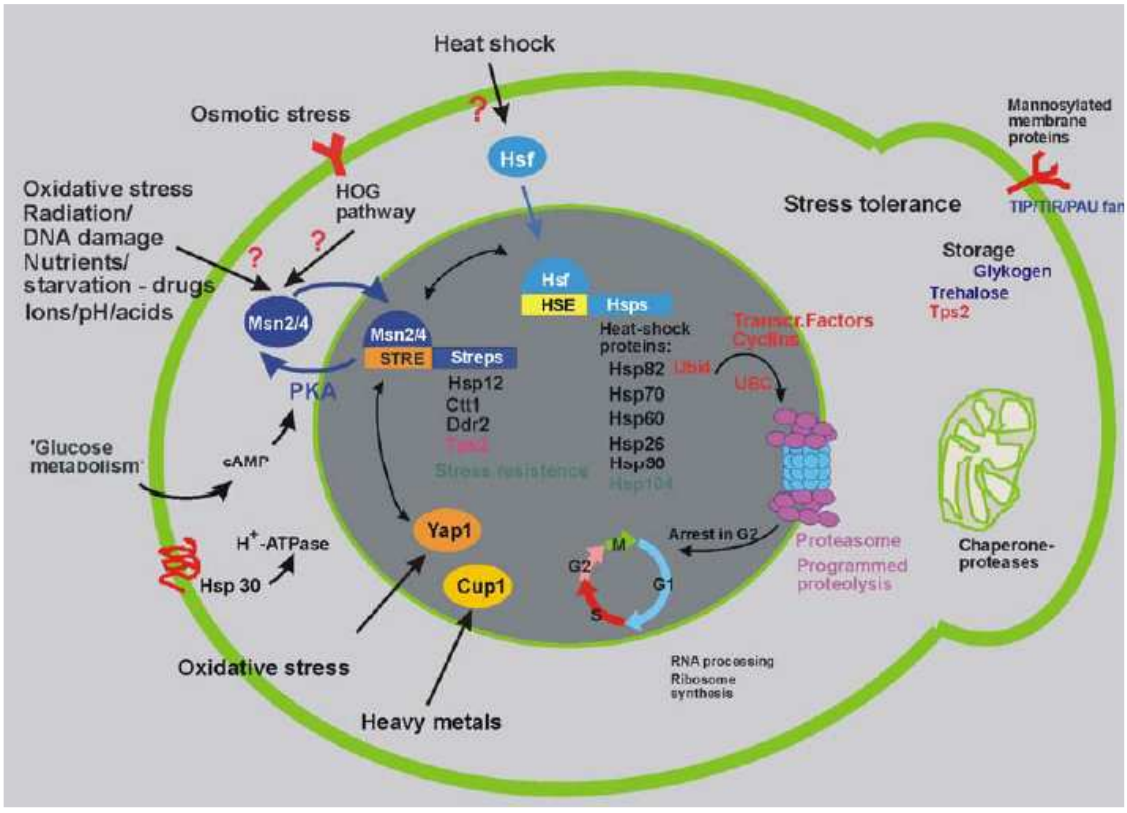

Figure 12: Overview on stress responses in yeasts [4]

From a basic point of view, to understand this response includes unravelling how the stress signal is sensed and transduced to the nucleus, to identify which genes are induced under each stress condition and, finally, to establish the phenotypic consequences of this induction in stress tolerance [36].

3.3.3. Sensing and signalling

Environmental stress and its primary cellular consequences (See chapter 3.2.2) are signals for cell that something is happening and that adequate response need to be elicited. Signal transduction pathways allow cells to respond to their environment and to change their behavior accordingly [15, 37].

Signals are sensed by receptors. Receptors are very specific, they are activated by only one signal. There are two types of receptors [37]:

1. soluble intracellular receptors

Soluble receptors sense intracellular signals. On binding a signal molecule, the soluble receptors change their conformation (are activated) and gain the ability to bind to specific DNA sequences in the nucleus and so induce transcription of this gene [37].

Some examples of this type of receptor is redox-sensitive signalling protein, transcription factor Yap1p which is activated in the appearance of free radicals and heat shock transcription factor Hsf1p which is activated by recruitment of heat shock protein [15].

2. *transmembrane receptors* (enzyme coupled receptors, G-protein coupled receptors and ion channel coupled receptors) [37]

Transmembrane receptors recognize extracellular signals and transmit the signal inside the cell. Binding to an extracellular receptor (receptor activation) activates many downstream targets and amplifies the signal. Amplification can occur by three mechanisms:

- producing a second messenger (cAMP)
- activating a G-protein
- activating a protein kinase

  - tyrosine kinases – the transmembrane receptor kinases or others that are recruited to a transmembrane receptor. They use ATP to phosphorylate specific tyrosine residues in the target protein.

  - serine/threonine protein kinases: PKA (A kinase; cAMP-dependent protein kinase), PKC (C kinase), MAP kinase (mitogen-activated protein kinase). They use ATP to phosphorylate specific serine or threonine residues in their targets. Not all serine or threonine residues in a target protein get phosphorylated. For each target protein there will be a specific pattern of phosphorylation that leads to the effect.

  The kinases can be arranged into phosphorylation cascades so that one kinase phosphorylates another, which, in turn, phosphorylates another. Depending on the particular target, phosphorylation may activate or inactivate it [37].

Table 3: The most important pathways in the ESR [31]

Signalling pathway	Signal
CWI pathway	secretion defects and cell wall damage
MEC1 pathway	DNA damage
Ssk1p/Ste11p-dependent pathway	osmotic stress
MAP kinase Hog1p	osmotic stress
PKA pathway	cAMP accumulation
TOR pathway	nutrient availability

Each of the pathways that control ESR gene expression is known to be activated by specific cellular signals, and consistently, most of the pathways tested govern ESR initiation only under the conditions that trigger their activity. Thus, despite the commonality of ESR initiation, the program is regulated by condition-specific mechanisms, allowing the cell to activate the ESR in response to a wide variety of upstream signals while maintaining specificity in how the cell senses and otherwise responds to each new environment [15, 31].

3.3.4. The genomics of yeast responses to environmental stress

An important aspect of each cellular response to environmental change is the reorganization of genomic expression to the program required for growth in new environment. Changes in ESR gene expression are proportional to the magnitude of environmental stress [15, 31, 38].

3.3.4.1. Genes afected by environmental stress

When cells are shifted to stress environments, they respond with changes in the expression of hundreds or thousands of genes, revealing the plasticity of genomic expression. Comparing the genomic expression programs elicited by different environmental transitions reveals that some of the expression changes are specific to each new environment, while others occur in all of the experiments tested and represent a common response to environmental stress [15].

- *a common response to environmental stress*

Comparative analysis of the genomic expression responses to diverse environmental changes revealed that the expression of roughly 900 genes (around 14% of the total number of yeast genes) is stereotypically altered following stressful environmental transitions. These genes fall into two groups based on their expression patterns [15, 31, 38]:

1. *Repressed genes* - around 600 genes whose transcripts are decreased in abundance following stressful environmental transitions. More than 70% of the characterized genes are involved in protein synthesis, including genes required for ribosome synthesis and processing, RNA polymerase I- and III dependent transcription, and protein translation. The reduced synthesis of these transcripts and their products may help to conserve energy while the cell adapts to its new conditions [15, 31, 38].
2. *Induced genes* - around 300 genes whose transcripts increase in abundance in response to the transitions. The genes whose expression is induced in the ESR are involved in a wide variety of cellular processes, including carbohydrate metabolism, protein folding and degradation, oxidative stress defense, autophagy, cytoskeletal reorganization, DNA-damage repair and other processes. The functions of these gene products may protect critical aspects of the internal milieu, such as energy reserves, the balance of the internal osmolarity and oxidation-reduction potential, and the integrity of cellular structures including proteins and DNA. The protection of these features by the ESR gene products likely contributes to the cross-resistance of yeast cells to multiple stresses, in which cells exposed to a mild dose of one stress become tolerant of an otherwise-lethal dose of a second stressful condition [15, 31, 38].

- *specificity of genomic expression programs*

In addition to triggering common ESR initiation, many of the environmental transitions provoke expression changes in highly specialized genes [15, 31, 38].

Table 4: Specific stress-induced genes [31, 38]

Environmental stress	Induced genes
Heat shock	heat shock proteins
Osmotic stress	enzymes of glycerol synthesis and sugar transporters
Oxidative stress	YAP1, ROS scavengers
DNA damage	RNR genes, Rad53

Under stress conditions, the expression of genes encoding functions important for stress adaptation is stimulated and their translation has to be ensured. This suggests that mechanisms must exist that allow the preferential translation of subsets of mRNAs under certain conditions [38].

3.3.4.2. Regulation of ESR gene expression

A cell can change the expression of its genes in response to external signals. Control of expression is vital to allow a cell to produce the gene products it needs. In turn this gives cells the flexibility to adapt to a variable environment, external signals, damage to the cell, etc. Regulation of ESR gene expression is condition-specific and a variety of regulatory mechanisms have been implicated in the regulation of ESR gene expression. Gene expression can be regulated at many of the steps in the pathway from DNA to RNA to protein (See *Figure 13*) [12, 15, 31, 38].

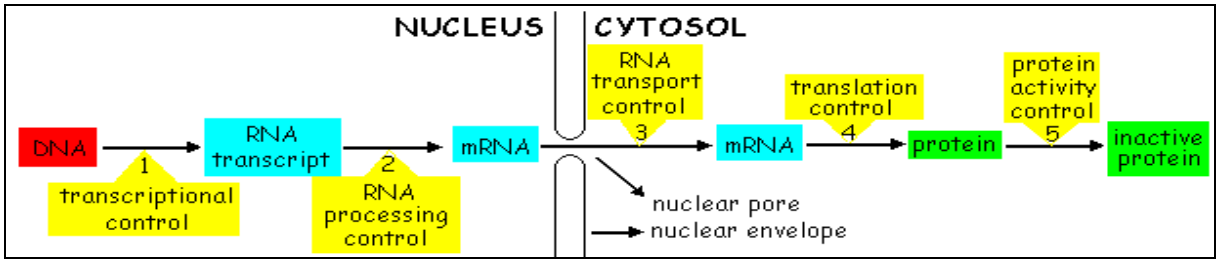

Figure 13: Eukaryotic gene expression can be controlled at several different steps [12]

The most studied regulation step in ESR is regulation of transcription. RNA polymerase binds to DNA at specific sites (promoters) to initiate transcription. A major site is the TATA box. For many genes, there are other DNA sequences that regulate transcription by binding specific proteins (transcription factors). The transcription factor binding sites may be located at varying distances from the transcription start site, and a given promoter region may be affected by more than one of these sites. The binding of transcription factors (enhancers and repressors) to a specific site on the DNA regulates the transcription by enhancing or inhibiting the formation of the complex structure that is required to initiate transcription [37, 39].
Two types of transcription factors are presented in ESR:

- "general stress" transcription factors *Msn2p* and *Msn4p* – transcription factors that are active in all cases of environmental stress [15, 31, 39, 40]
- *condition-specific transcriptional factors* - transcription factors that control ESR gene expression only under specific conditions: the transcription factors Hsf1p, Hot1p, and Yap1p independently affect the expression of subsets of ESR genes in response to heat shock, osmotic shock, or oxidative stress, respectively, but are uninvolved in regulating the expression of these ESR genes under other conditions [15].
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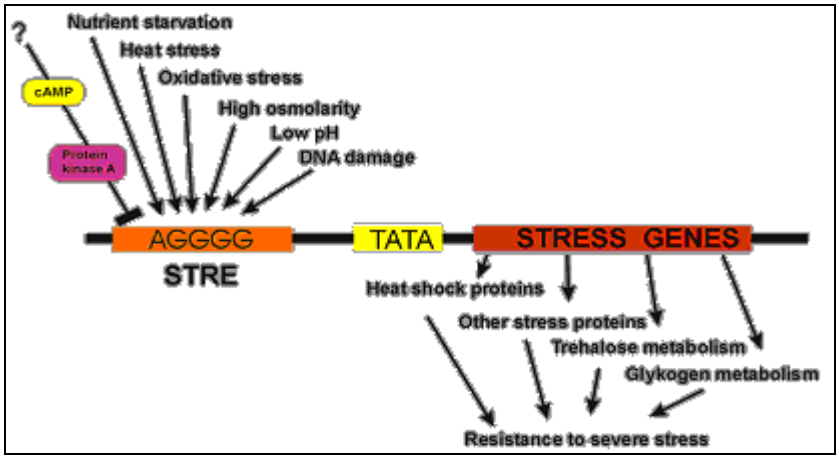


Figure 14: Factors controlling stress response elements (STREs) and effects triggered by STRE activation in yeast [2]

3.3.5. Physiological/phenotypic consequences of ESR

Altered genome expression results in alteration in the abundance of the corresponding gene products. Indeed, many of the changes in ESR transcript levels correlate with changes in protein synthesis. Proteomic studies have identified proteins whose translation increases or decreases following starvation, osmotic shock, oxidative stress and heat shock. Therefore, despite the overall decrease in protein translation following a stressful environmental transition, the cell is still able to translate proteins that are critical for its survival. The transient changes in gene expression may help the cell to rapidly adjust the concentrations of the corresponding gene products to the levels required for growth at the new conditions. The genes induced in the ESR are involved in a wide variety of processes (See *Table 5*) [15].

Table 5: Physiological responses of yeasts to environmental stress [2]

General response	Examples
Thermoprotection	HSP biosynthesis, decreased membrane lipid unsaturation, altered cell pH, polyamine biosynthesis
Cryoprotection	enhanced trehalose accumulation, increased membrane lipid polyunsaturation
Osmoprotection	accumulation of compatible solutes (glycerol, trehalose), increased K+ uptake/Na+ efflux
Antidesiccation	trehalose accumulation
Antioxidation	enzymatic (superoxide dismutase, catalase, cytochrome peroxidase), non-enzymatic (glutathione, thioredoxin, metallothionein, polyamines, carotenoids)
Detoxification	ethanol: stress proteins, altered membrane transport, mitochondrial superoxide dismutase, xenobiotics: glutathione heavy metals: stress proteins, metallothioneins

3.3.5.1. Growth arrest

One of the most important consequence of the ESR is the coordinated control of the progression of the cell cycle. In response to environmental stress, signal pathways mediate a transient cell cycle arrest required for proper cell adaptation. For instance, during cell cycle arrest, cells are able to modify their transcriptional program which allows the cell to save energy and to adapt to new conditions [15, 41].

Many of the starvation-specific expression alterations may be rationalized by the fact that starvation involves a transition from active growth to growth arrest, in contrast to the response to other stresses in which cells resume growth after adapting to the new conditions [38].

3.3.5.2. Cytoskeletal reorganization

When actively growing cells progress through the cell cycle, the arrangement of their actin cytoskeleton is polarized so that vesicles and materials can be delivered along the actin cables to the site of cell growth at the bud neck. However, in response to cellular stress, the actin cytoskeleton becomes rapidly depolarized and instead arranges isotropically, perhaps to deliver secretory vesicles and their cargo evenly within the cell. Soon after the adaptation to the new environment, the cytoskeleton becomes repolarized to promote localized growth and subsequent cell division. Reorganization of the cytoskeleton may be affected by factors encoded by genes in the ESR [15, 42].

3.3.5.3. The heat shock proteins or Hsps

All organisms respond to potentially hazardous environment by synthesizing a set of proteins that protect them from damage and facilitate recovery from such stress. These gene products are classically known as heat shock proteins (HSPs) and are highly conserved across evolutionary lineages [15, 43].

Heat shock proteins act like 'chaperones', making sure that the cell's proteins are in the right shape and in the right place at the right time. For example, HSPs help new or distorted proteins fold into shape, which is essential for their function. They also shuttle proteins from one compartment to another inside the cell, and target irreparable proteins for degradation. Heat shock proteins are also believed to play a role in the presentation of pieces of proteins (or peptides) on the cell surface to help the immune system recognize diseased cells [43].

Heat shock proteins are classified by their molecular weight, size, structure, and function. They are divided into several families: HSP110/104 [44]; HSP90 [45]; HSP70 [15, 44]; HSP60 (chaperonin) [44] and small heat shock proteins [44].

3.3.5.4. Antioxidant defence

Yeast cells require to maintain a reduced intracellular state when faced with oxidative stress and they possess various antioxidant chemicals and enzymes to detoxify active oxygen. These molecules are capable of removing oxygen radicals and their products and/or repairing the damage caused by oxidative stress [2, 15, 36, 46].

Non-enzymatic antioxidant defence

- *Glutathione*

  Perhaps the best-known example of a non-enzymatic defence system is glutathione (GSH), a tripeptide γ-L-glutamyl-L-cystinylglycine. GSH acts as a radical scavenger with the redoxactive sulphydryl group reacting with oxidants to produce reduced glutathione (GSSG). Glutathione is possibly the most abundant redox scavenging molecule in cells, consequently, its role in maintaining cellular redox state is important [36, 46, 47].
- *Polyamines*

  Amino acid-derived polyamines (spermine and spermidine) have also been implicated in protecting yeast against oxidative stress [46].
- *Thioredoxin*

  Thioredoxin is a small sulphydryl-rich protein with a conserved sequence (Trp-Cys-Gly-Pro-Cys) in its active site. When reduced, the dithiol group of the active site is able to catalyse the reduction of disulfites in a number of proteins. The thioredoxin system is composed by thioredoxin (Trx), thioredoxin reductase (Trr) and NADPH and in yeast there are two of such systems, one in the cytoplasm with two thioredoxins (Trx1p and Trx2p) and one thioredoxin reductase (Trr1p) and a second in the mitochondria with one thioredoxin (Trx3p) and one thioredoxin reductase(Trr2p) [36, 46, 47].
- *Glutaredoxin*

  Glutaredoxins are a class of small proteins with an active site containing redox sensitive cysteines. These proteins are thought to act in much the same way as thioredoxins. Yeast contains glutaredoxins with two subfamilies that differ in the number of cysteine residues at the active site. The first subfamily protects cells against H_2O_2 (Grx2p) and superoxide anions (Grx1p). The second family includes three additional members (Glx3p, Glx4p and Glx5p) and, among them, Grx5p seems to play an important role in protection against oxidative stress, both during ordinary growth conditions and after exposure to oxidants such as H_2O_2 and menadione [36, 46].

- *Metallothioneins*

  Metallothioneins are a class of small cysteine-rich proteins with antioxidant properties and have the capacity to bind a number of different metal ions. Metallothioneins are particularly important in countering the toxicity of metals such as copper. They have been also shown to play a role in protecting yeast cells against oxidants and confer menadione resistance [46, 48].
- Other antioxidat chemicals: carotenoids (See chapter 3.4.1.2.), ascorbic acid, phytochelatins, flavonoids, etc. [46]

Enzymatic antioxidant defence

- *Catalase*

  Catalase catalyses the breakdown of H_2O_2 to O_2 and H_2O. *S. cerevisiae* has two such enzymes, catalase A and catalase T, encoded by the CTA1 and CTT1 genes, respectively. Catalase A is located in the peroxisome and the main physiological role of this enzyme appears to be to remove H_2O_2 produced by fatty acid beta-oxidation. The physiological role of the cytosolic catalase T protein is less clear; CTT1 gene expression is, however, regulated by oxidative and osmotic stress and by starvation [36, 46, 47].
- *Superoxide dismutase*

  Yeast cells possess two intracellular superoxide dismutases: the mitochondrially-located Mn-SOD (encoded by the SOD2 gene) and the cytoplasmically-located Cu/Zn-SOD (encoded by the SOD1 gene). Superoxide dismutases disproportionate two molecules of superoxide anion to H_2O_2 and O_2. The Cu/Zn-SOD appears to be the major enzyme involved in removing superoxide anions from the cytoplasm and possibly also the peroxisome and there is also some evidence for a role of the Cu/Zn-SOD in protecting cells against respiration derived superoxide anions. The Mn-SOD appears to protect the mitochondria from superoxides generated during respiration and exposure to ethanol, and seems to play little role in countering the toxicity of superoxide anions generated by exogenously added redox cycling compounds during fermentative growth [36, 46, 47].
- *Glutathione reductase and glutathione peroxidase*

  Crucial to the role of glutathione as an antioxidant is the maintenance of a high reduced–oxidized ratio inside the cell. The enzyme glutathione reductase is primarily responsible for the reduction of oxidized glutathione and maintenance of the GSH/GSSG ratio in cells. The enzyme glutathione peroxidase catalyses the reduction of hydroperoxides, using GSH as a reductant. Yeast glutathione peroxidase activity towards both H_2O_2 and organic hydroperoxides has been demonstrated; interestingly, both these activities could be induced by a shift from anaerobic to aerobic growth conditions [46].
- *Thioredoxin peroxidase and thioredoxin reductase*

  Thioredoxin peroxidase reduces both H_2O_2 and alkyl hydroperoxides, in conjunction with thioredoxin reductase, thioredoxin and NADPH. Thioredoxin peroxidase levels are increased about two-fold in response to growth in the presence of 95% O_2 or thiol-containing agents such as mercaptoethanol [46, 47].
- *Methionine reductase*

  Methionine residues exposed on the surface of proteins can act as antioxidants to protect the active sites of proteins. Methionine residues on the surface of an enzyme would be oxidized to methionine sulphoxide, effectively removing the oxidant and protecting the active site of the protein. The enzyme methionine sulphoxide reductase would then reverse the process [46, 48].

3.3.5.5. Carbohydrate metabolism

A critical component of cell survival is maintaining a viable energy source. Glucose is the preferred carbon source in yeast, and upon stress, the cell induces a variety of genes that affect glucose metabolism. This includes genes encoding glucose transporters that serve to import external glucose into the cell and glucose kinases that activate the sugar for subsequent catabolism. In response to stressful environments, the fate of glucose is divided between trehalose synthesis, glycogen storage, ATP synthesis through glycolysis, and NADPH regeneration by the pentose phosphate shuttle [15].

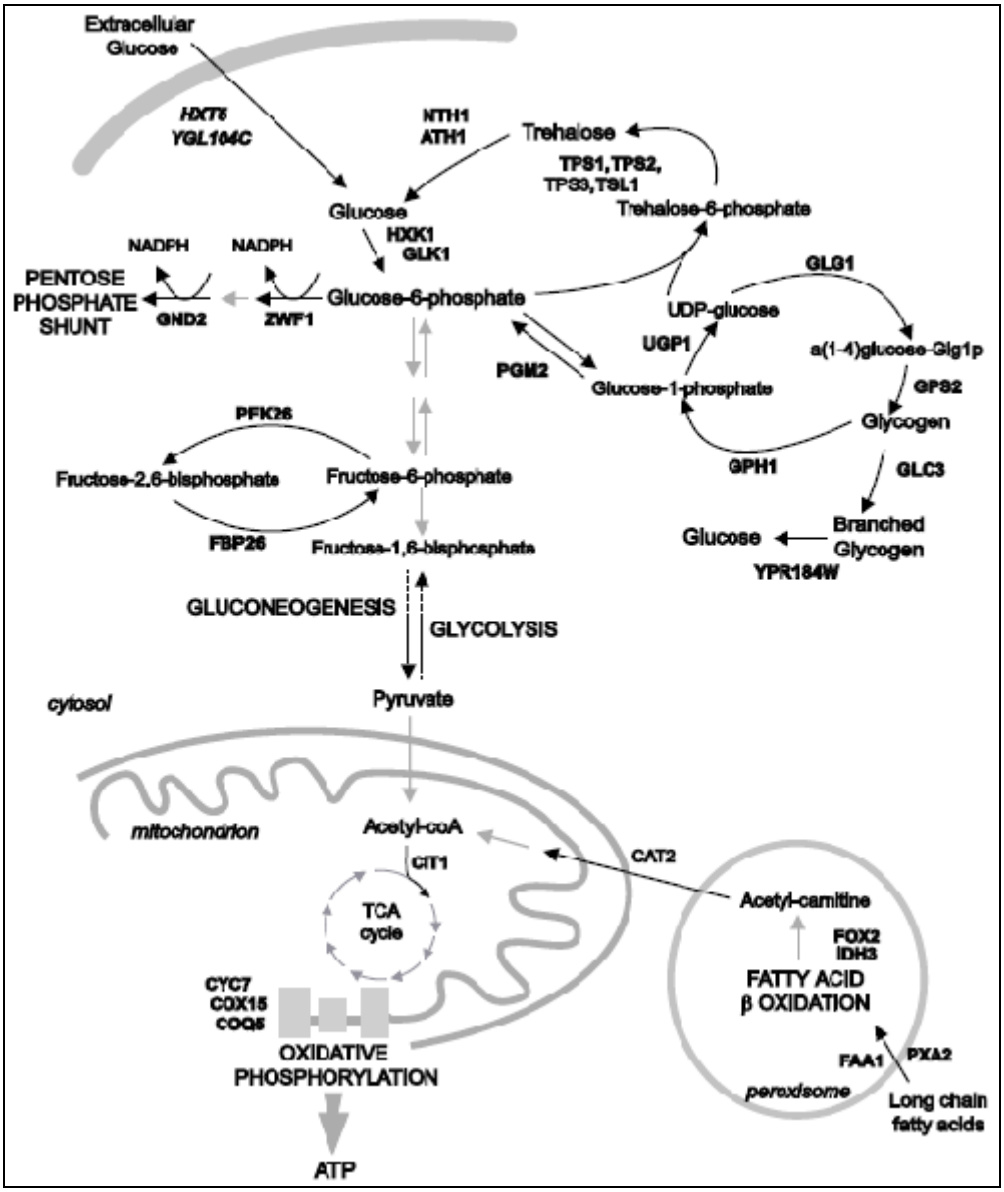


Figure 15: ESR genes involved in carbohydrate metabolism [15]

Glycogen and trehalose are typical hallmarks of rapid adaptations of yeast cells to environmental changes [15, 49].

Glycogen is a high molecular mass branched polysaccharide of linear α (1,4)-glucosyl chains with α (1,6)-linkages. The major function of glycogen is to provide carbon and energy for maintenance of cellular activities when nutrients are scarce. It likely plays an important role in response to a wide variety of stressful environments as well [49].

Trehalose is a non-reducing disaccharide composed of two α (1,1)-linked glucose molecules. It has long been implicated in stress responses, particularly the response to hyperosmolarity, heat shock, and oxidative stress. A high content of trehalose protects cells from autolysis. Trehalose fulfils two unique properties that make this molecule a stress protectant: (1) it protects membranes from desiccation („the water-replacement hypothesis“) and (2) excludes water from the protein surface and hence protects proteins from denaturation in hydrated cells. Therefore, both trehalose and the Hsp104 chaperone are required for refolding proteins in the cytosol and also for conformational repair of heat-damaged glycoprotein in the endoplasmatic reticulum lumen. This synergy between trehalose and some Hsps in stress tolerance is effective only if the trehalose accumulation in response to stress is followed by its rapid mobilization as soon as the cells return to normal conditions [15, 49].

The precise levels of glycogen and trehalose within the cell is controlled by modulation of activities of enzymes that promote the synthesis and degradation of glycogen and trehalose. Genes encoding these enzymes are coinduced in the ESR and are posttranslationally regulated by phosphorylation and by allosteric regulation [15, 49].

3.3.5.6. Fatty acids metabolism

Yeast cells can consume metabolites other than glucose to generate energy, including fatty acids. Beta-oxidation of fatty acids occurs in the yeast peroxisome, and the resulting acetyl moieties are then transported to the mitochondria where they supply the TCA cycle for anabolic metabolism and respiration. Although most of the genes involved in fatty acid metabolism are not induced in the ESR, genes involved in importing (*FAA1*, *PXA2*) and exporting (*CAT2*) these metabolites into and out of the peroxisome are induced. The induction of these genes may facilitate fatty acid oxidation simply by increasing the local concentration of fatty acid substrates while efficiently removing the metabolic products for further catabolism in mitochondria. This would allow the cell to take full advantage of existing peroxisomes, rather than expend significant energy required to proliferate the organelles [15].

3.3.5.7. Respiration

In the presence of glucose, yeast cells are unique among microbes in that they rely on fermentation to generate ATP, despite the fact that respiration generates many more ATP molecules per glucose. Genes involved in respiration are normally repressed in the presence of glucose, however they can be derepressed by a reduced ATP:AMP ratio in the cell, even in the presence of the sugar. As many of the stress defense mechanisms consume ATP, it might be expected that many types of cellular stress would lead to the induced expression of respiration components. While this is true of the response to environmental changes that lead to substantial cellular stress, the expression of genes encoding respiration components is not induced under all circumstances, and these genes have an expression profile subtly distinct from the ESR expression pattern. However, as part of the ESR, the cell does induce a handful of genes that have been implicated in respiration. For example, in response to diverse stresses the cell induces genes encoding the rate-limiting step of the TCA cycle (*CIT1*), an alternate isoform of cytochrome c (*CYC7*), and two factors that affect the synthesis and assembly of the oxidative phosphorylation components cytochrome c oxidase (*COX15*) and ubiquinone (*COQ5*). Increasing the levels of these gene products may promote ATP synthesis by utilizing existing respiration components. Alternatively, the induction of factors that promote cytochrome c and ubiquinone synthesis may play a role in the defense against oxidative stress [15, 38].

3.3.6. Study of ESR

Functional analysis of the genome and proteom provides the tools for understanding the roles of gene products, their expression patterns, and how they interact to create a eukaryotic organism capable of complex processes like growth, cell division, and the response to extracellular signals. A number of different experimental approaches have been used to study gene expression in cells responding to different environments, and each method presents different features of stress responses. DNA microarrays, two-dimensional gel electrophoresis and mass spectrometry-driven proteomics can be used to identify genes and proteins involved in environmental stress responses [31].

3.3.6.1. DNA microarrays – identification of genes involved in ESR

DNA microarray analysis is essentially a method for carrying out thousands of hybridizations at one time using smalls samples. DNA probes representing each of the *Saccharomyces* ORFs are irreversibly attached to a solid substrate such as a glass slide or a nylon membrane. The unique sequence fragments are made by PCR using carefully selected primer pairs internal to the transcribed regions. The DNA fragments are spotted onto the substrate using specialized devices capable of producing an 80 x 80 array of 6400 samples in an area of about 18 mm^2. The DNA sample under analysis is incubated with the DNA chip under conditions that allow hybridization. The sample DNA is labeled with a fluorescent tag and laser scanning or a fluorescent confocal microscope detects positions of hybridization. The results are expressed quantitatively relative to a control condition and changes of twofold or more are considered significant [50].

DNA microarrays can be used to assess a variety of cellular features, including transcript abundance of essentialy every gene in an organism's genome, localization, and polysome association, as well as the presence and location of DNA-binding proteins bound throughout the genome. Characterizing environmentally triggered gene expression changes provides insights into when, where, and how each gene is expressed and offers a glimpse at the physiological response of the cells to changes in their surroundings [31, 51].

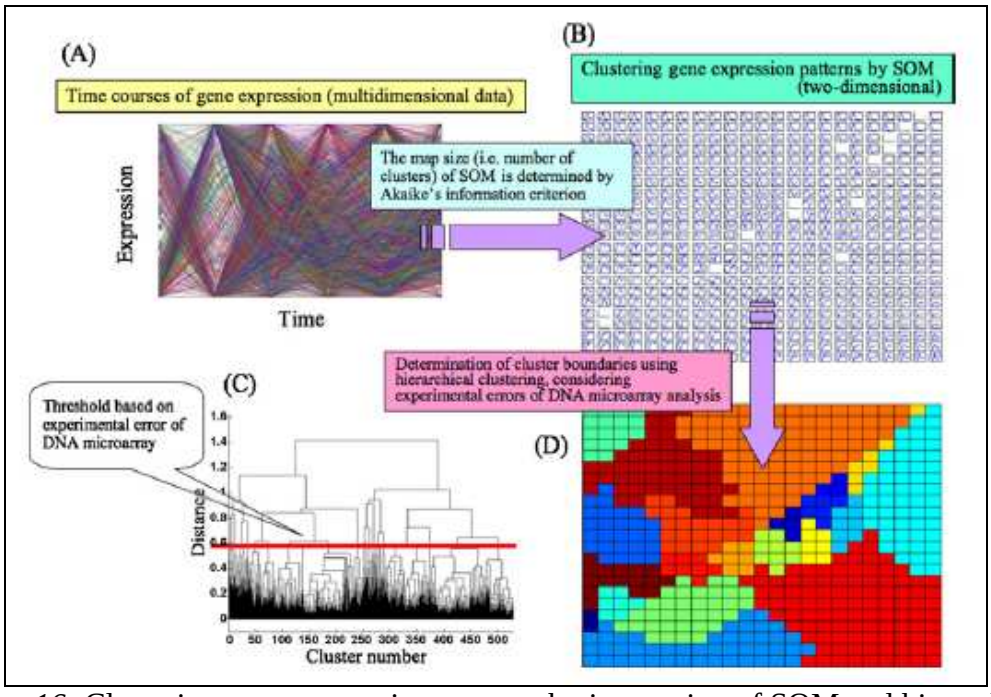


Figure 16: Clustering gene expression patterns by integration of SOM and hierarchical clustering

3.3.6.2. Two-dimensional gel electrophoresis and mass spectrometry – identification of proteins involved in ESR

The proteomes of cells are extremely complex, consisting of several thousand proteins. Because of this complexity, twodimensional polyacrylamide gel electrophoresis (2DE) has been widely used as the standard protein separation and display method [11].

Two-dimensional gel electrophoresis exploit a combination of two different single dimension runs. Two-dimensional maps could be prepared by using virtually any combination of 1D methods. The most useful two-dimensional gel electrophoresis separates proteins according to two independent parameters, isoelectric point (pI) in the first dimension and molecular mass (Mr) in the second dimension by coupling isoelectric focusing (IEF) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The first dimension is preferably performed in individual IPG strips laid side by side on a cooling platform, with the sample often adsorbed into the entire strip during rehydration. At the end of the IEF step, the strips have to be interfaced with the second dimension, almost exclusively performed by mass discrimination via saturation with the anionic surfactant SDS. After the equilibration step, the strip is embedded on top of an SDS-PAGE slab, where the 2D run is carried out perpendicular to the 1D migration. The 2D map displayed at the end of these steps is the stained SDS-PAGE slab, where polypeptides are seen, after staining, as spots, each characterised by an individual set of pI/Mr coordinates. Proteins separated on 2D gels are visualised by either staining with Coomassie blue dye, silver stains, fluorescent dyes, immunological detection or by radiolabelling and quantifying using densitometers, fluoro- and/or phosphorimagers. Theoretically, 2DE is capable of resolving upto 10,000 proteins simultaneously, with approximately 2000 proteins being routine, and detecting and quantifying protein amounts of less than 1 ng per spot. The position of a spot in the 2D map is not the enough information for an exact identification of a protein. For routine analysis, protein spots of interest (e.g. up- or down-regulated proteins) are excised from the 2D gel, digested into fragments by specific proteases and then identified using mass spectrometry (MS) and database mining [11].

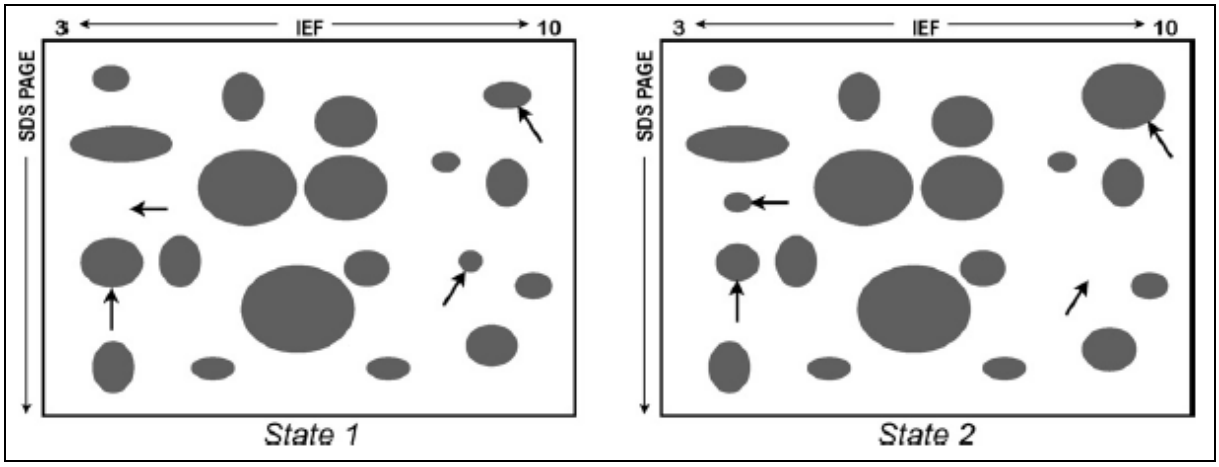


Figure 17: Schematic representation of 2D gels showing different stages of a proteome. Up and down regulated genes (quantitative and qualitative changes) are marked with arrows [11].

A comprehensive description of the proteome of an organism not only provides a catalogue of all proteins encoded by the genome but also data on protein expression under defined conditions [11].

3.3.6.3. Gene disruption/deletion – functional analysis of genes

Gene disruption/deletion is a method by which a DNA fragment is used to replace a genome sequence with a selectable marker gene (See *Figure 18*). With the complete yeast sequence in hand it became possible to use PCR-based methods to delete each of the ORFs [51, 52].

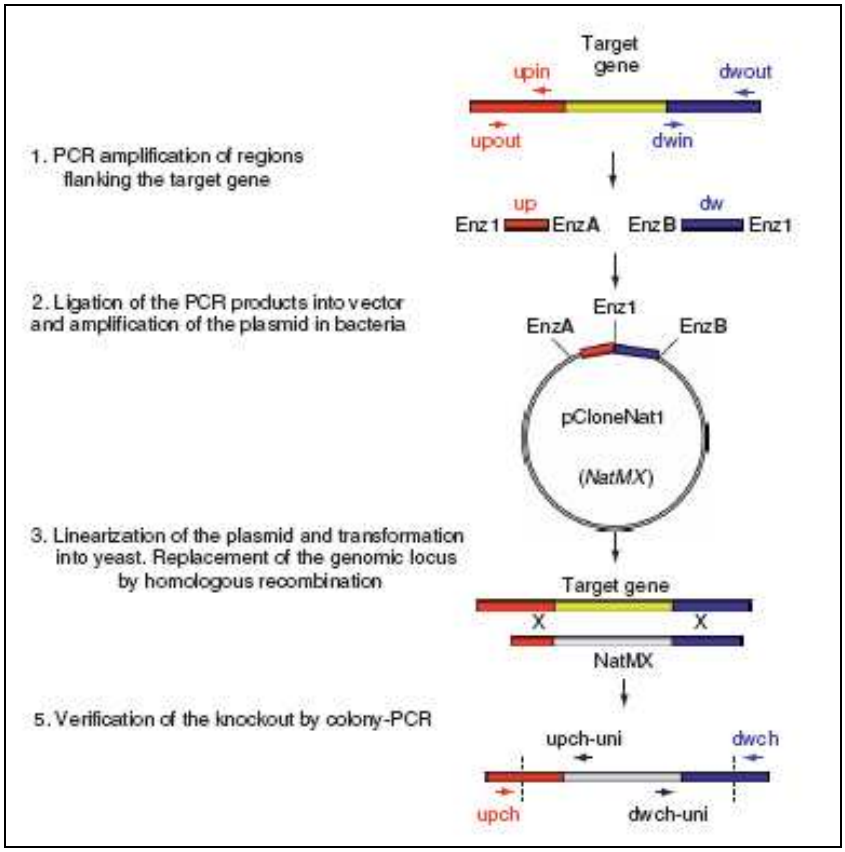


Figure 18: Flowchart of the knockout strategy [52]

When deleting a gene, it is best to remove sequences starting in the promoter and extending into the ORF or past the stop codon. This ensures that the gene has been functionally knocked out. If the transcription and translation start sites are not removed and the deletion is internal to the ORF, it is conceivable that some gene function could be retained [51].

The availability of the yeast deletion set with target gene deletions makes it possible to identify, among all the nonessential genes in yeast, those genes whose products are required for specific cellular processes or contribute to stress resistance in a dose-dependent manner [31].

Ultimately, the integration of genomic data with data obtained by more classical methods will lead to the ability not only to predict but also to modify and possibly construct novel responses in yeast and other organisms [31].

3.4. CAROTENOGENIC YEASTS AND ENVIRONMENT

3.4.1. Carotenogenic yeasts

3.4.1.1. General characteristics of carotenogenic yeasts

Some yeast species accumulate carotenoid pigments, such as β-carotene, torulene, and thorularodin which cause their yellow, orange and red colours and are therefore called red yeasts. Carotenogenic yeasts are a diverse group of unrelated organisms (mostly *Basidiomycota*) and the majority of the known species are distributed in four taxonomic groups: the *Sporidiobolales* and *Erythrobasidium* clade of the class *Urediniomycetes*, and *Cystofilobasidiales* and *Tremellales* of the class *Hymenomycetes* [53].

- *Rhodotorula species*

  The genus *Rhodotorula* includes three active species; *Rhodotorula glutinis*, *Rhodotorula minuta* and *Rhodotorula mucilaginosa* (formerly known as *Rhodotorula rubra*) [54].

  Colonies are rapid growing, smooth, glistening or dull, sometimes roughened, soft and mucoid. They are cream to pink, coral red, orange or yellow in color. Blastoconidia that are unicellular, and globose to elongate in shape are observed. These blastoconidia may be encapsulated. Pseudohyphae are absent or rudimentary. Hyphae are absent [54].

  Rhodotorula glutinis is a free living, non-fermenting, unicellular yeast found commonly in nature. Rhodotorula is well known for its characteristic carotenoids “torulene, torularhodin and β-carotene. *Rhodotorula glutinis* is also reported to accumulate considerable amount of lipid [55].

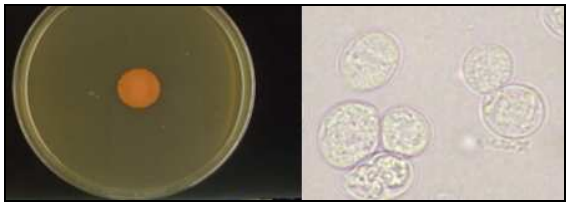


Figure 19: Rhodotorula glutinis

- *Sporobolomyces species*

  The genus *Sporobolomyces* contains about 20 species. The most common one is *Sporobolomyces roseus* and *Sporobolomyces salmonicolor* [54].

  Sporobolomyces colonies grow rapidly and mature in about 5 days. The optimal growth temperature is 25-30°C. The colonies are smooth, often wrinkled, and glistening to dull. The bright red to orange color of the colonies is typical and may resemble *Rhodotorula spp*. [54].

  Sporobolomyces produces yeast-like cells, pseudohyphae, true hyphae, and ballistoconidia. The yeast-like cells (blastoconidia, 2-12 x 3-35 µm) are the most common type of conidia and are oval to elongate in shape. Pseudohyphae and true hyphae are often abundant and well-developed. Ballistoconidia are one-celled, usually reniform (kidney-shaped), and are forcibly discharged from denticles located on ovoid to elongate vegetative cells [54].

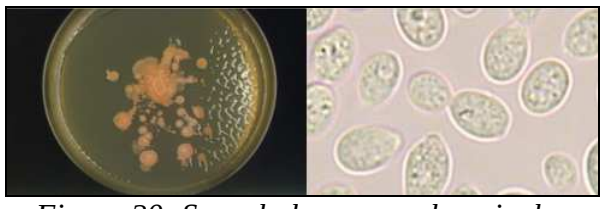


Figure 20: Sporobolomyces salmonicolor

3.4.1.2. Carotenoids as a biotechnologically significant metabolites of red yeasts

Carotenoids are the most pronounced, naturally occurring pigments. They are of great interest in many scientific disciplines because of wide distribution and diverse functions. Owing to their ubiquitous occurrence, many functions and interesting properties,carotenoids are subject of interdisciplinary research in biochemistry, biology, chemistry, medicine, microbiology, physics and many other branches of science [56].

Chemical structure of carotenoids

Carotenoids are class of hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls). They consist of eight isoprenoid units joined in such a manner that the arrangement of isoprenoid units is reversed at the center of the molecule. The two central methyl groups are in 1,6-relationship and the remaining non-terminal methyl groups are in a 1,5-position relationship. Most carotenoids contain a linear C_{40} hydrocarbon backbone that includes between 3 and 15 conjugated double bonds [56, 57].

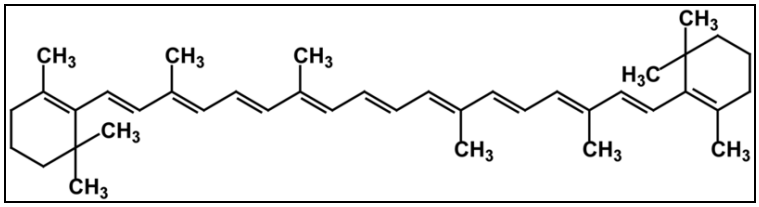


Figure 21: Chemical structure of β-carotene [57]

Physical properties of carotenoids

Carotenoids have characteristic absorption spectra because of presence of conjugated ployene system in the molecule and distinct functional groups. The number of double bonds largely determines the spectral properties of a given carotenoid, which typically absorbs light between 400 and 500 nm. The increase in the position of the absorption maxima due to an additional conjugated double bond varies from 7 to 35 nm. Insertion of other functional group twists the chromophore out of plane and reduces both fine structure and intensity of spectra (hypochromic effect) [57, 58].

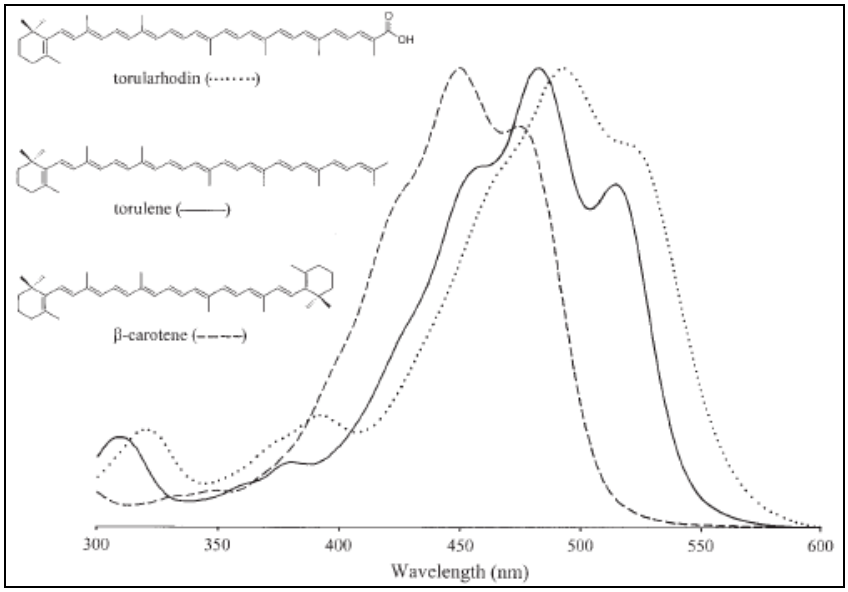


Figure 22: Absorption spectra and molecular structures of the three major pigments of *Sporobolomyces roseus* [59]

Biosynthesis of carotenoids

Carotenoids are synthesized in nature by plants and many microorganisms. Acetyl-CoA was demonstrated as the key precursor of carotenoid biosynthesis. Carotenoids biosynthesis pathway commonly involves three steps: (i) formation of isopentyl pyrophosphate (IPP), (ii) formation of phytoene and (iii) cyclization and other reactions of lycopene [57, 60].

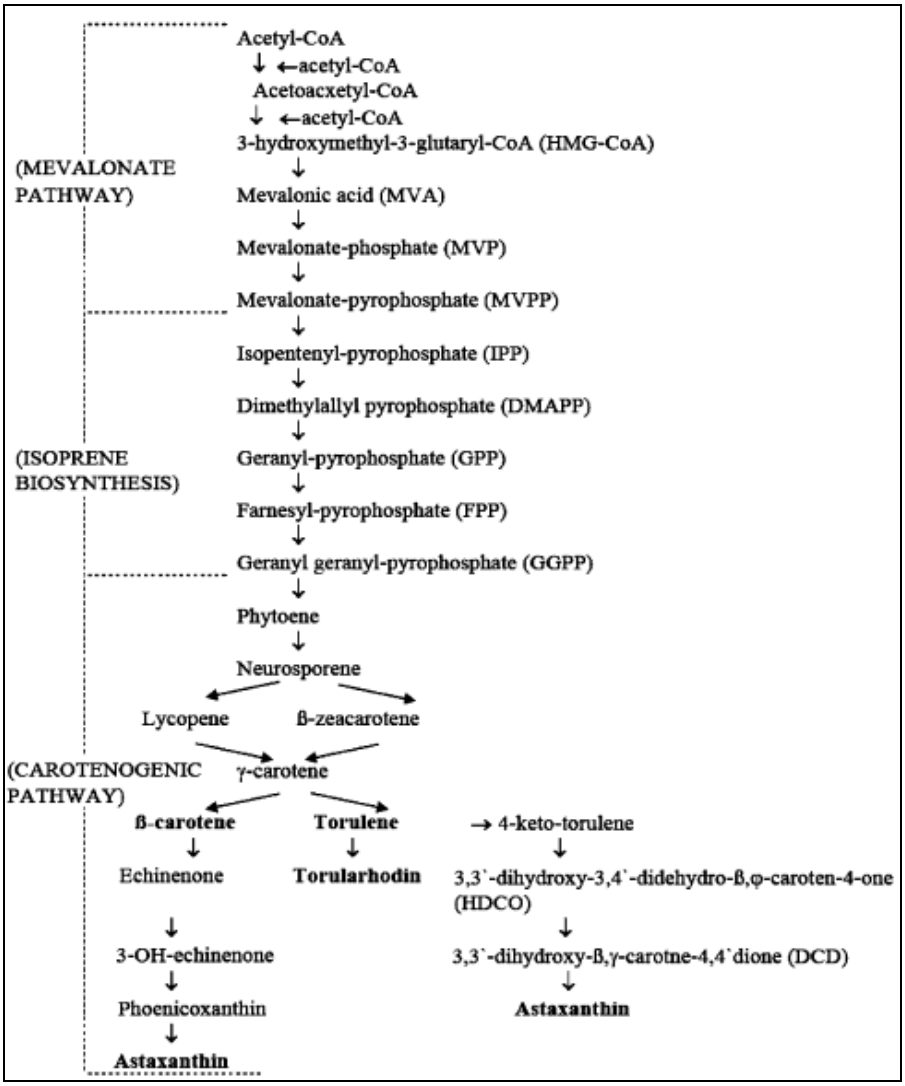


Figure 23: Biosynthetic pathways from acetyl-CoA to β-carotene, torulene and torularhodin in *Rhodotorula* species and astaxanthin in *P. rhodozyma/X. dendrorhous* [61]

Functions of carotenoids

Carotenoids have the ability to act as antioxidants and thus protect cells against photooxidation. The ability of carotenoids to quench singlet oxygen is well known and reactions with radical species have also been studied [62]. Dietary carotenoids inhibit onset of many diseases in which free radicals are thought to play a role in initiation, such as atherosclerosis, cataracts, age-related macular degeneration, multiple sclerosis and most importantly cancer [63]. Antioxidant properties of carotenoids are exploited also in cosmetics. A cosmetic preparations comprising the carotenoids were reported to be effective in preventing various kinds of damage resulting from oxidation and exposure to UV light [56]. Carotenoids are popular also in food industries as colourants and vitamin A sources [64]. Carotenoid-containing preparations are also playing important role as feed additive. Astaxanthin is the major carotenoid used for pigmentation of fishes and salmons [65].

Carotenoid analysis

High-performance liquid chromatography is regarded as the preferred method for the separation, identification, and quantitation of carotenoids found in biological samples. Unfortunately, complex mixtures of carotenoids, many of which are closely related structurally, are often present in biological matrices, rendering unequivocal identification by HPLC using retention time and fixed wavelength data alone unacceptable. The advent of photodiode array detection, allowing for continuous collection of spectral data during HPLC analysis, has provided a powerful tool for carotenoid research. However, tentative identification of carotenoids using HPLC with photodiode array detection requires retention time measurement, complete chromatographic resolution of absorbing species so that spectrophotometric data for the analyte alone are observed, and comparison of UV/VIS spectra and retention times with those of authentic standards. Mass spectrometric and tandem mass spectrometric analyses, which provide molecular weight and characteristic fragmentation patterns, may then provide final confirmation of individual carotenoid identities when used in conjunction with retention and spectral characteristics [66-69].

Several LC-MS techniques have been used for carotenoid analysis:

LC/MS Technique	Molecular Ion Species	Extent of Fragmentation	Limit of Detection
Moving belt	$M^{+\cdot}$	very high	poor (ref. 5)
Particle beam	$M^{+\cdot}$	medium	not reported
CF-FAB	$M^{+\cdot}$	low-none	9-28 pmol (ref. 10)
Electrospray	$M^{+\cdot}$	low-none	1-2 pmol (ref. 11)
APCI	$M^{+\cdot}$, $[M+H]^+$	low for carotenes	3-13 pmol (ref. 13)
	$M^{-\cdot}$, $[M-H]^-$	medium for xanthophylls	1-3 pmol (ref. 13)

Figure 24: Comparison of LC/MS techniques used for carotenoid analysis [67]

- atmospheric pressure chemical ionization (APCI)

  APCI takes place at atmospheric pressure and uses a heated nebulizer to facilitate solvent evaporation and obtain a fine spray of the HPLC mobile phase. The analyte is „spraying“ into a corona discharge, which produces numerous reactive species that can ionize the analyte including reagent gas ions formed from the mobile phase [70].

  The first APCI LC/MS analyses of carotenoids show some unexpected molecular ion heterogeneity. Molecular ions and protonated molecules were observed in positive ion APCI, and molecular ions and deprotonated molecules were detected during negative ion APCI. The relative abundance of molecular ions and protonated or deprotonated molecules vary with the mobile phase composition. For instance, polar solvents such as alcohols lead to an increased abundance of protonated carotenoids (even protonated β-carotene), and less polar solvents such as methyl-tert-butyl ether facilitate the formation of more abundant molecular ions. The limit of detection, ease of use, compatibility with HPLC solvents and flow rates, and suitability of APCI for automated or unattended LC-MS carotenoid analysis are comparable to those of electrospray. The main advantage of APCI compared to electrospray is its higher linearity of detector response. Disadvantages of APCI include the multiplicity of molecular ion species, which might lead to ambiguous molecular weight determinations, and the abundant fragmentation, which tends to reduce the abundance of the molecular ions [67, 70].

- electrospray (ESI)

  Electrospray is both an ionization technique and an efficient solvent removal interface. During electrospray, a fine mist of charged droplets is formed from the HPLC eluate at atmospheric pressure by spraying the solution through a capillary electrode at high potential (usually 2,000-7,000 V). As the charged droplets are electrostatically attracted towards the opening of the mass spectrometer, they encounter a cross-flow of heated nitrogen curtain gas that increases solvent evaporation and prevents most of the solvent molecules from entering the mass spectrometer. When the droplets shrink in size until the electrostatic repulsion between ions in each droplet exceeds the combined energy of solvation and surface tension, ions are ejected from the droplets into the gas phase or else the droplet disintegrates and releases analyte ions. Because of the efficiency of this combined ionization and desolvation process, electrospray is compatible with HPLC flow rates from a few μl/min up to 1 ml/min [67].

  In carotenoid analysis, negative ion electrospray mass spectrometry may be used to detect polar xanthophylls, but positive ion electrospray LC-MS was shown to be useful for the analysis of both xanthophylls and carotenes. Although ions produced by electrospray are usually preformed in solution by acid/base reactions, carotenoid ions are probably formed by a field desorption mechanism at the surface of the droplet. In order to enhance the formation of molecular ions, solution-phase carotenoid oxidation was carried out by means of postcolumn addition of a halogenated solvent to the HPLC effluent (such as chloroform; 2,2,3,3,4,4,4-heptatluoro-1-butanol; 2,2,3,3,4,4,4-heptafluorobutyric acid; 1,1,1,3,3,3- hexafluoro-2-propanol or trifiuoroacetic acid). As a result of this unusual ionization process, electrospray of carotenoids produces abundant molecular cations, M^+, with little fragmentation, and no molecular anions [66, 67].

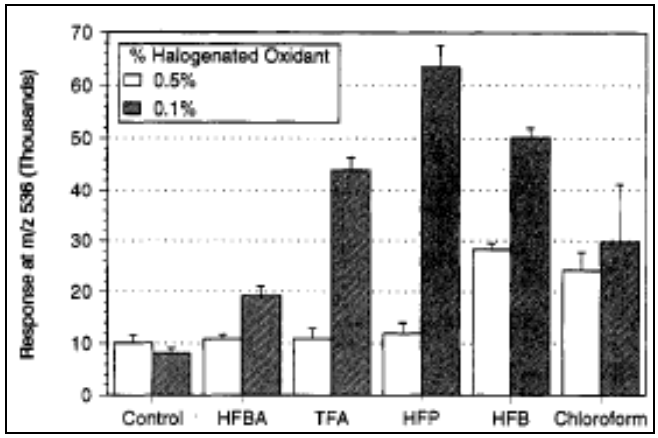


Figure 25: Comparison of halogenated solvents as oxidants during PI ESI MS of β-carotene [66]

Advantages of electrospray LC/MS technique for carotenoids include lower limits of detection, abundant molecular ions of both xanthophylls and carotenes, substantially improved ease of operation, reduced maintenance, higher sample throughput, and compatibility with autosamplers for unattended operation. One of the potential limitations of electrospray is the relatively narrow range of linearity of response it shows for carotenoid quantitation [67].

3.4.2. Carotenogenic yeast and environment

Carotenogenic yeasts are considered to be ubiquitous due to its world-wide distribution in terrestrial, freshwater and marine habitats, and to its ability to colonize a large variety of substrates. They can assimilate various carbon sources, such as glucose, xylose, cellobiose, sucrose, glycerol, sorbitol, etc. For this reason, various waste materials can be used as cheap substrates for its cultivation. The red yeast is able to grow under a wide range of initial pH conditions from 2.5 to 9.5 and over a wide range of temperatures from 5 to 26°C [61, 71, 72].

Under stress conditions the red yeast accumulates higher quantity of carotenoids. This is of increased interest to the biotechnology. The use of this stressed biomass in feed industry could have positive effect not only in animal and fish feeds because of high content of physiologically active substances, but it could influence nutritional value and organoleptic properties of final products for human nutrition. Knowledge of molecular mechanism of the carotenoid production stimulation can then lead to improvement of such biotechnological process [61, 73].

3.4.2.1. Effect of environmental stress on protein synthesis

Under stress conditions expression of some protein fractions is changed. Significant 2D proteome changes were observed mainly in peroxide stress (underproduction of 247 from 310 evaluated proteins). Relatively well-balanced response was observed in presence of 2 % salt; it seems that *R.glutinis* cells could exhibit long-term adaptation to this stress factor. With increased salt concentration 67 spots exhibited gradual quantitative changes in the same direction [73].

More detailed protein results can be found in simultaneously measured work of Halienova: *Changes in production of proteins and other metabolites of selected organisms in stress conditions.*

3.4.2.2. Effect of environmental stress on growth

Addition of stress factors into cultivation medium led to different changes of growth according to the yeast species, type of stress factor or growth phase, in which stress factors were added. Addition of H_2O_2 caused in most strains substantially greater biomass production decrease than did the addition of NaCl. This decrease was lower, however, when H_2O_2 was added during the exponential phase of growth (expect in the case of *Sporidiobolus* and *R. aurantiaca*). On the other hand, growth of all tested strains with expection of the *R. glutinis* in medium with 5-10% NaCl decreased only slightly [74].

3.4.2.3. Environmental stress and carotenoid production

The most important consenquence of environmental stress in red yeast is stimulation of carotenoid production. Carotenoid pigments accumulation in most yeasts starts in the late logarithmic phase and continues in the stationary phase and is highly variable. Carotenoid production depends on differences between strains of the same species and is strongly influenced by the cultivation conditions [61].

- substrates for carotenoid production

  The presence of a suitable carbon source is important for carotenoid biosynthesis. *Rhodotorula* species have potential commercial value as a dietary source of natural carotenoids; however, the high cost of production limits the use of these yeasts. Different agro-industrial raw materials that create serious environmental problems may be possibly used as low-cost carbohydrate sources, with the view of minimizing production cost and environmental problems [61, 75-77].

Various natural substrates were tested as carbon sources for carotenoid production, such as: grape must [75]; hydrolyzed mung bean waste flour [78]; sugar cane and sugar-beet molasses [76, 79]; corn syrup [80]; milk whey [61]; etc.

Table 6: Comparison of cell mass and carotenoid production by *Rhodotorula species* grown on different waste substrates as carbon sources

Rhodotorula species	Carbon source	Cultivation process	Cell mass (g/l)	Carotenoids (mg/g CDW)	Carotenoids (mg/l culture)	References
R. glutinis ATCC 26085	glucose	batch		0.206		[81]
R. glutinis 32	glucose	batch	23.9	5.4	129.0	[79]
R. glutinis 32	sugar cane molasses	fed-batch	78.0	2.36	183.0	[79]
R. glutinis DBVPG 3853 *D. castellii* DBVPG 3503	corn syrup	fed-batch	15.3	0.535	8.20	[80]
R. glutinis TISTR	hydrolyzed mung bean waste flour	batch	10.35	0.345	3.48	[78]
R. glutinis 22P *L. helveticus* 12A	whey ultrafiltrate	batch	30.2	0.268	8.10	[61]
R. mucilaginosa NRRL-2502	sugar-beet molasses	batch	4.2	21.20	89.0	[76]
R. mucilaginosa NRRR-2502	whey	batch	2.4	29.2	70.0	[76]

- effect of light

  Carotenogenesis in many organisms is regulated by light. However, the intensity and protocol of illumination varies with the microorganism. The cultivation of the yeast in the dark and under sunlight conditions showed that light was not a limiting factor for growth and pigmentation of *R. glutinis* DFR-PDY. In contrast, light stimulated carotenogenesis in many other microorganisms, such as *Phaffia rhodozyma*, where cultures exposed to light synthesized more astaxanthin with a redder hue [61, 72].

- effect of temperature

  Temperature is another important factor affecting the performance of cells and product formation. The effect of temperature depends on the species specificity of the microorganism and often manifests itself in quantity variations of synthesized carotenoids. It was reported that lower temperatures (25°C) seemed to favor synthesis of β-carotene and torulene, whereas higher temperatures (35°C) positively influenced torularhodin synthesis by *R. glutinis* [61, 75].

- effect of aeration and oxidative stress

  Carotenogenesis is an aerobic process. The effect of aeration is dependent on the species of the microorganism. The reported optimal values of air flow rate and agitation are in range 0.5 – 1.9 l/min and 180 – 900 rpm for carotenogenesis in yeasts *Rhodotorula*. The aeration influenced not only the amount of carotenoids produced, but also the composition of individual pigments making up the total carotenoids. At higher aeration, the concentration of total carotenoids increased relative to the biomass and fatty acids in *R. glutinis*, but the composition of carotenoids (torulene > β-carotene > γ-carotene > torularhodin) remained unaltered. In contrast, *S. roseus* responds to enhanced aeration by a shift from the predominant β-carotene to torulene and torularhodin [61, 81].

Also other induces of oxidative stress such as illumination or free radical generators have a significant effect on the carotenoid production. By UV mutagenesis of the pink yeast *R. glutinis* the yellow colored mutant 32 was obtained which produced 24-fold more total carotenoids (2.9 mg/g dry cells) and 120-fold more β-carotene (2.05 mg/g dry cells) than the wild-type in a much shorter time (36 hours) [61, 82]. A sevenfold increase in β-carotene accumulation was reported for yeast mutant *R. glutinis* 10 EMS which was obtained after treatment with 10 mg/ml of ethyl-methane sulfonate. Production of carotenoids by *Rhodotorula glutinis* cells grown under oxidative stress was about 5–6 times higher than in wild-type [74].

- osmotic stress

  Divalent cation salts increased the total carotenoid content about two times. It can be assumed that this positive response was due to a stimulatory effect of cations on carotenoid-synthesizing enzymes or to the generation of active oxygen radicals in the culture broth [61].

4. OBJECTIVES

Carotenogenic yeasts are well known producers of valuable carotenoid pigments. The carotenoid rich biomass of red yeast is widely used in feed industry. Carotenoid production by fermentation can become feasible if the cost of production can be minimized. To decrease the cost of the biotechnological process various waste substrates can be used as carbon or nitrogen source.

The first objective of this PhD project so was:

I. to investigate growth and carotenoid production by red yeasts cultivated on various waste materials, such as: whey, potato mass, apple mass or various cereal-based substrates

The cost of the biotechnoligical process can be decrease also by stimulation of carotenoid production. Environmental stress (oxidative, osmotic, irradiation...) was reported to increase the carotenoid production in some carotenogenic yeasts. Knowledge of molecular mechanism of the carotenoid production stimulation can then lead to improvement of such biotechnological process.

The second objective of this PhD project so was:

II. to study intracellular changes caused by the environmental stress, i.e. to study:
   i. effect of environmental stress on yeast growth
   ii. effect of environmental stress on cell morphology
   iii. effect of environmental stress on ROS production
   iv. effect of environmental stress on DNA integrity

The next step of the molecular study of carotenoid production stimulation by environmental stress is to identify genes and their products accountable for this stimulation. For this reason a protocol for preparation of knock-out strains need to be first designed.

The third objective of this PhD project so was:

III. to design a strategy for preparation of strains with deletion of definete genes – the protocol was firstly set up for genetically more simple yeast *Schizosaccharomyces pombe*

5. MATERIALS AND METHODS

5.1. Yeast strains

Saccharomyces cerevisiae CCM 8191
Schizosaccharomyces pombe sp286
Rhodotorula glutinis CCY 20-2-26
Rhodotorula rubra CCY 20-7-31
Rhodotorula aurantiaca CCY 20-9-7
Sporobolomyces roseus CCY 19-6-4
Sporobolomyces shibatanus CCY 19-20-3
Cystofilobasidium capitatum CCY 10-1-1
Cystofilobasidium capitatum CCY 10-1-2

Carotenogenic yeasts were obtained from the Culture Collection of Yeast (CCY), Bratislava, Slovakia.

5.2. Bacterial strain

Competent *Escherichia coli* DH5α cells

5.3. Chemicals

Chemicals for yeast cultivation

glucose anhydrous p.a., Lachema (CZ); yeast extract, Scharlau (Spain); L-leucine, Sigma (DE); L-lysine hydrochloride, Sigma (DE); L-histidine, Sigma (DE); uracil, Sigma (DE); adenine, Sigma (DE); G418 sulfate (Geneticin®), Serva (DE); ammonium sulfate p.a., Lachema (CZ); potassium dihydrogenphosphate p.a., Lachema (CZ); magnesium sulfate heptahydrate p.a., Chemapol (CZ); yeast autolysate, Himedia (India); hydrogene peroxide, Fluka (DE); sodium chloride p.a., Lachema (CZ); agar powder, Himedia (India); whey, Pribina, a.s, Přibyslav (CZ); potato extract, Himedia (India); potatos, apples, apple fiber, pastes, wheatmeal and whole-grain frumenty – from hypermarket or healthy food shops (CZ); wheatbran – mill (CZ); grains – brewery (CZ)

Chemicals for ROS determination

dihydroethidium, Sigma-Aldrich (DE); 2‘,7‘-dichlorodihydrofluorescein diacetate, Sigma-Aldrich (DE)

Chemicals for chromosome isolation and PFGE

Chelaton 3 (EDTA) p.a., Lachema (CZ); Tris(hydroxymethyl)aminomethane (Tris), Serva (DE); dodecylsulfate Na-salt (SDS), Serva (DE); Proteinase K, Serva (DE); lyticase, Sigma-Aldrich (DE); low melting point agarose, Serva (DE); β-mercaptoethanol, Serva (DE); N-lauroyl sarcosine, Sigma-Aldrich (DE); boric acid, Lachner (DE); agarose PREMIUM, Serva (DE); ethidium bromide, Serva (DE); Yeast Chromosome PFG Marker (16 chromosomes isolated from *Saccharomyces cerevisiae*, strain YPH80; size range: 225-1,900 kb), BioLabs (USA)

Chemicals for carotenoid and ergosterol extraction and analysis

acetone p.a., Lachema (CZ); potassium hydroxide; diethyl ether; ethanol UV p.a., Lachema (CZ); ethanol for UV-VIS, Riedel-de Haën (DE); methanol for HPLC, Sigma-Aldrich (DE); acetonitrile for HPLC, Sigma-Aldrich (DE); β-carotene (β,β-carotene), Sigma (DE), xanthophyll (α-Carotene-3,3′-diol, β, ε-carotene-3,3′-diol, β, ε-carotene, lutein), Sigma (DE); ergosterol, Sigma (DE)

Chemicals for gene knockout

DreamTaq DNA Polymerase: DreamTaq DNA Polymerase (5u/μl), 10 × DreamTaq Buffer, 25mM $MgCl_2$, Fermentas (DE); dNTP Mix, Fermentas (DE); primers (upout, upin, dwout, dwin, upch, dwch), Takara; restriction enzymes (XbaI, NheI, BglII, XhoI, BamHI, HindIII, SalI, SspBI), Fermentas (DE); T4 DNA ligase, Fermentas (DE); QIAquick PCR purification kit, QIAGEN (DE); QIAquick PCR purification kit, QIAGEN (DE)

Chemicals for yeast transformation

salmon sperm DNA, Stratagene (DE); lithium acetate, Aldrich (DE); polyethylene glycol 4000, Serva (DE)

5.4. Equipment

Analytical balance AND HR 120 (GB)
Laboratory balance
Autoclave
Laminar flow box Aura Mini (Bio Air Instruments, Italy)
Shaker LT 2, Kavalier Glass Works (CZ)
Shaking incubator Heidolph, Promax (SRN)
Shaking incubator Heidolph, UNIMAX (SRN)
Heating incubator
Adjustable pipettes
Adjustable 0.5 – 10 and 30 – 300 μl eight-channel pipettes (Eppendorf)
System for light microscopy:

- Light microscope, LII00a (Germany)
- GKB Color digital CCD Camera (Taiwan)
- Software Lucia Image Active 5.0., Laboratory Imaging Ltd. (CZ)

System for fluorescence microscopy:

- Fluorescence microscope Leica DMR XA2 (Leica Microsystems, GmbH Germany)
- CoolSNAP CCD camera (Roper Scientific, GmbH Germany)
- Openlab software (Improvision, Germany)

Camera Polaroid DS 34 (Japan)
Water bath EL-20 (Merci a.s., CZ)
Spectrophotometer VIS Helios δ (UNICAM, UK)
Spectrophotometr UV-VIS Helios α (UNICAM, UK)
PFGE equipment:

- Gene Navigator (Pharmacia Biotech, Sweden)
- GN Controller (Pharmacia Biotech, Sweden)
- Programmable Power Supply, MP-500V (Sweden)
- Thermostatic cooler (Julabo Labortechnic GmbH, Germany)

Gel documentation system:

- UltraLum, Inc. Electronic Dual Wave Transilluminator (USA)
- UltraLum, Inc. Ultra Viewer (USA)
- UltraCam CCD (USA)
- UV-Transilluminator, TVR-312A (Spectroline, USA)

Vortex TK 3s, Kartell (Italy)
Centrifuge 3-15 (Sigma, Germany)
Refrigerated centrifuge, MLW K24 (Germany)
Rotary vacuum evaporator RVO-64 (CZ)

HPLC system from ECOM Ltd. (CZ)

- Gradient programer GP 5
- Pump P 4020
- Analytical Injection Loop Valve D
- Column oven LCO 101
- UV-VIS detector LCD 2084
- Clarity (DataApex) SW and HW for HPLC data collection and evaluation
- Nucleosil100 C18 column, 150 x 4.6 mm, 7 mm with guard column 30 x 4.6 mm, 7 mm

HPLC/PDA/ESI-MS:

- Gradient Surveyor MS Pump Plus (Thermo Finnigan, USA)
- Photo Diode Array detector PDA Surveyor Plus (Thermo Finnigan, USA)
- Analytical Injection Loop Valve D (ECOM, CZ)
- Column oven LCO 101 (ECOM, CZ)
- Vacuum pump (Thermo Finnigan, USA)
- MS detektor LCQ Advantage Max (Thermo Finnigan, USA)
- LS-MA SW Xcalibur 1.3. (Thermo Finnigan, USA)

Qiaprep 96 turbo miniprep kit (Qiagen)

PCR tubes

96-well plates for PCR (with cover stickers)

PCR machines

Electrophoresis unit (Bio-Rad)

5.5. Cultivation of *Saccharomyces cerevisiae*

5.5.1. Cultivation media

Budding yeast *S. cerevisiae* was grown in liquid YPD medium (See *Table 7*).

Table 7: YPD medium composition

Components	(g/l dH_2O)
glucose	20
yeast extract	10
peptone	20

5.5.2. Cultivation conditions

Cells were cultivated in flasks at 30°C in a rotary shaker with vigorous aeration (150 rpm).

5.5.3. Maintainance of *Saccharomyces cerevisiae*

Yeast *S. cerevisiae* was maintained on malt agar slants at 4°C.

5.6. Cultivation of *Schizosaccharomyces pombe*

5.6.1. Cultivation media

Fission yeast *Sz. pombe* was grown in YES, a rich yeast extract-based medium (See *Table 8*).

Table 8: YES medium composition

Components	(g/l dH_2O)
glucose	30.00
yeast extract	5.00
L-leucine	0.10
L-lysine hydrochlorid	0.10
L-histidine	0.10
uracil	0.10
adenine sulphate	0.15

5.6.2. Cultivation conditions

Cells were cultivated in flasks at 32°C in a rotary shaker with vigorous aeration (200 rpm).

5.6.3. Maintainance of *Schizosaccharomyces pombe*

Sz. pombe was stored in liquid YES medium with 50 % glycerol at -80°C.

5.7. Cultivation of carotenogenic yeast

All used carotenogenic yeast strains (See chapter 5.1.) were cultivated in glucose media of the same composition and at the same cultivation conditions (see below).

5.7.1. Cultivation media

In experiments, the yeast cells were grown in liquid simple glucose media. For cultivation of carotenogenic yeasts two types of simple glucose media were used: inoculation medium and production medium (See *Table 9* and *Table 10*).

Table 9: Inoculation media (I, II)

Components	(g/l tap H_2O)
glucose	40
yeast autolysate	7
$(NH_4)_2SO_4$	5
KH_2PO_4	5
$MgSO_4$	0.34

Table 10: Production medium

Components	(g/l tap H_2O)
glucose	40
$(NH_4)_2SO_4$	4
KH_2PO_4	4
$MgSO_4$	0.34

5.7.2. Cultivation conditions

Each red strain was cultivated at optimal growth conditions: aerobically at 28°C, at permanent lighting and shaking at 120 rpm; in two-steps inoculation: all strains were firstly inoculated into INO I, cultivated for 24 hours and transfered into INO II. Cultivation in INO II underwent for 24 hours and cells were then poured into production media. Cultivation in production media was done for 80 hours (for carotenoid analysis), 48 hours (for protoplast preparation), 75 hours (for chromosome analysis) or 24 hours (for ROS evaluation).

5.7.3. Maintainance of carotenogenic yeasts

Cultures of each carotenogenic yeast strain were maintained on malt agar slopes and YPD agar plates at 4°C.

5.7.4. Stress treatments

Red yeast cells were treated with hydrogen peroxide (2; 5; 10 and 100 mM; for induction of oxidative stress) or NaCl (2; 5 and 10 %; for induction of osmotic stress) under the conditions indicated above. Exogenous stress factors were added into production media at the beginning of the cultivation.

5.7.5. Cultivation on various waste substrates

Each of tested carotenogenic strains was cultivated at optimal growth conditions and in medium with modified carbon and nitrogen sources. For studies on the effect of other carbon sources, glucose was replaced by the indicated carbon sources. Similarly yeast extract was also replaced by the indicated nitrogen source. As a carbon or nitrogene source various waste substrates were used in this study; such as whey, potato mass, apple mass and different cereal-based substrates. They were added either into INO II or into production media or into the both (See *Table 12*, *Table 13*, *Table 14* and *Table 15*).

Table 11: Whey composition

Components	Sweet whey (g/l)	Sour whey (g/l)
Dry weight	63.0 – 70.0	63.0 – 70.0
Lactose	46.0 – 52.0	44.0 – 46.0
Proteins	6.0 – 10.0	6.0 – 8.0
Calcium	0.4 – 0.6	1.2 – 1.6
Phosphates	1.0 – 3.0	2.0 – 4.5
Lactate	2.0	6.4
Chlorides	1.1	1.1
pH	6.1	4.6

Whey pretreatment:

- Whey liquid - Wl (See *Table 11*)
- Whey lyophillized - WL

  Liquid whey was lyophilized on a laboratory lyophilizer and used as nutrient source.
- Whey lyophilized, acidified - WLA

  Acidified deproteinated whey was prepared from liquid whey. The pH of liquid whey was adjusted to 4.6 with 0.1 M H_2SO_4 and it was held in boiling water bath for 20 min for protein denaturation. After denaturation, the solution was cooled to laboratory temperature and the precipitate was removed by centrifugation. The supernatant was lyophilized on a laboratory lyophilizer and used as nutrient source.

Table 12: Cultivation on whey and potato extract

		1st series	2nd series	3rd series
INO I		See *Table 9*		
INO II (40g glucose, 5g $(NH_4)_2SO_4$, 5g KH_2PO_4, 0.34g $MgSO_4$)		yeast autolysate (7 g/l)	whey (7 g/l)	potato extract (7 g/l)
PRODUCTION (See *Table 10*)	wt	-	-	-
	Ma	2% NaCl	2% NaCl	2% NaCl
	Mb	5% NaCl	5% NaCl	5% NaCl
	WL	whey lyophillized non-processed (7 g/l)	whey lyophillized non-processed (7 g/l)	
	WLA	whey lyophillized processed (acidified) (7 g/l)	whey lyophillized processed (acidified) (7 g/l)	
	Wl	liquid whey (volume of medium)	liquid whey (volume of medium)	
	PE	potato extract (7 g/l)		potato extract (7 g/l)

Table 13: Cultivation on potato mass

		1st series	2nd series
INO I		See *Table 9*	
INO II (40g glucose, 5g $(NH_4)_2SO_4$, 5g KH_2PO_4, 0.34g $MgSO_4$)		yeast autolysate (7 g/l)	potato extract (7 g/l)
PRODUCTION (4g $(NH_4)_2SO_4$, 4g KH_2PO_4, 0.34g $MgSO_4$)	wt/p	glucose (40 g/l)	
	G-PE	glucose (20 g/l) + potato extract (20 g/l)	
	PE	potato extract (40 g/l)	
	G-PP	glucose (20 g/l) + potato peels (20 g/l)	
	PP	potato peels (40 g/l)	

Table 14: Cultivation on apple mass

		1st series	2nd series
INO I		See *Table 9*	
INO II (40g glucose, 5g $(NH_4)_2SO_4$, 5g KH_2PO_4, 0.34g $MgSO_4$)		yeast autolysate (7 g/l)	apple fiber (7 g/l)
PRODUCTION (4g $(NH_4)_2SO_4$, 4g KH_2PO_4, 0.34g $MgSO_4$)	wt/a	glucose (40 g/l)	
	G-AF	glucose (20 g/l) + apple fiber (20 g/l)	
	AF	apple fiber (40 g/l)	
	CA	crushed apple (40 g/l)	
	AP	apple peels (40 g/l)	

Table 15: Cultivation on various cereal-based substrates

		without enzyme pretreatement	with enzyme pretreatment
INO I		See *Table 9*	
INO II		See *Table 9*	
PRODUCTION (4g $(NH_4)_2SO_4$, 4g KH_2PO_4, 0.34g $MgSO_4$)	wt/c	glucose (40 g/l)	
	Pastes	paste (40 g/l)	
	AF	apple fiber (40 g/l)	
	WGF	whole-grain frumenty (40 g/l)	
	Grains	grains (40 g/l)	
	Bran	bran (40 g/l)	

* samples description: medium description (wt, WL, WLA, Wl, PE, See *Table 12*; wt/p, G-PE, PE, G-PP, PP, See *Table 13*; wt/a, G-AF, AF, CA, AP, See *Table 14* and wt/c, Pastes, AF, WGF, Grains and Bran, See *Table 15*) + number of series

Pretreatment of cereal substrates

Cereal materials mentioned in *Table 15* contain complex hydrocarbones which are not utilisable by red yeast. To increase their potential as carbon source, they were treated with extracellularly produced hydrolytic enzymes of mould *Fusarium solani*. *Fusarium solani* was grown on saline glucose medium for 24 hours and produced enzyme solution was frozen and lyophilized on a laboratory lyophilizer for 48 hours. Media containing selected cereal materials (See *Table 15*) (2 g in 50 ml of distilled H_2O) were incubated with lyophilized enzyme solution at laboratory temperature for 162 hours. Content of produced sugar was assayed at defined time points by HPLC and by the method of Somogyiho-Nelson. Samples were first hydrolyzed by 4 M HCl and then injected onto column Zorbax (4.6 × 150 mm) with aminophase (5 μm). Separation was carried out by isocratic elution at 25 °C. The mobile phase used was acetonitril:H_2O (75:25) with a flow rate of 1.0 ml/min. Detection was performed using refractometric detector.

5.8. Biomass evaluation and sample collection

Cell density was determined by turbidity measurements using a spectrophotometer at 630 nm and correlated to dry cell weight (CDW). The dry cell mass concentration was determined from the optical density reading by using the following equation:

- *Rhodotorula glutinis*: OD_{630} = 0.195×CDW (g/l) - 0.023
- *Rhodotorula rubra*: OD_{630} = 0.230×CDW (g/l)
- *Rhodotorula aurantiaca*: OD_{630} = 0.154×CDW (g/l) + 0.098
- *Sporobolomyces roseus*: OD_{630} = 0.242×CDW (g/l) - 0.049
- *Sporobolomyces shibatanus:* OD_{630} = 0.205×CDW (g/l) - 0.018
- *Cystofilobasidium capitatum:* OD_{630} = 1.470×CDW (g/l) + 0.876

Biomass was collected by centrifugation at 5000 rpm for 10 min at room temperature. Each cell pellet was resuspended in physiological solution and stored until used.

5.9. Growth determination

Growth of yeast was determined by sequentially assessing optical density of each culture at defined time points. All determinations of growth properties were done twice.

5.10. Carotenoid extraction and analysis

5.10.1. Extraction of carotenoids from yeast cells

For carotenoid isolation the whole biomass obtained from 50 ml of medium was used. Yeast cells were disintegrated using a mechanical disruption by shaking in a grinding mortar. A mixture of pigments and other organic compounds was extracted from the cell homogenate using 50 ml of acetone. After saponification of the extract by 10 % ethanolic KOH (30 min. at 90°C) carotenoids were extracted three times with 50 ml of diethyl ether. The diethyl ether extracts were collected and evaporated under vacuum to dryness. After evaporation, the residue was dissolved in absolute ethanol and analysed by RP–HPLC/ESI-MS.

5.10.2. Analysis of carotenoids and ergosterol by RP-HPLC/UV-VIS

Carotenoid pigments extracted from yeast cells were individually identified and quantified by RP-HPLC using a chromatographic system ECOM. Samples were filtered through PTFE filters and injected onto column. Separation was carried out by isocratic elution at 45 °C. The mobile phase used was methanol with a flow rate of 1.0 ml/min. Detection of β-carotene was achieved photometrically at 450 nm. Data processing (integration) of analyses was assessed using a CSW Integrator v.1.7m and Clarity SW (Data Apex Co.). Individual carotenoids were quantified using external standard of β-carotene (See chapter 5.3.; concentration range 10 – 200 μg/ml).

Ergosterol was analysed in the same filtered extract as carotenoids. Separation was carried out in the same column as carotenoids using a mixture of acetonitril/methanol 95:5 as mobile phase and UV detection at 285 nm for identification. Data processing of analyses was assessed using Clarity (DataApex) software. Ergosterol was quantified using external standard of ergosterol (See chapter 5.3.; concentration range 0.1 - 1 mg/mL).

5.10.3. Identification of carotenoids by RP-HPLC/PDA-ESI-MS

Identification of individual carotenoids were performed by LC/PDA-ESI-MS analysis. Carotenoid separation was carried out in the same way as described above (See chapter 5.10.2.). The PDA was used to record chromatograms simultaneously at wavelengths of 450, 350 and 290 nm and to determine peak spectra for carotenoid identiÞcation and for the checking of peak purity.

Individual carotenoids were analysed by on-line RP-HPLC/PDA/ESI-MS (Detector PDA Finnigan Surveyor, MS detector LCQ Advantage Max, Thermo Finnigan) using column type, mobile phase and chromatography conditions as described above. Analysis was performed at the flow rate 0.5 mL/min, mass spectra were analyzed by on-line ESI ionization in negative mode.

MS analysis was performed off-line too: separeted fractions of carotenoid mixture were collected into eppendorf tubes, mixed with chloroform (50:50, v/v) and injected directly into the LCQ Advantage Max ion-trap mass spectrometer with electrospray ionization (ESI). Positive ion ESI-MS spectra were monitored in the mass range of m/z 400–600. The capillary temperature was set to 150°C, the capillary voltage was optimized to 16 V and the sheath nitrogen gas flow was set to 30 (arbitrary units).

5.11. Microscopic determination of morphological changes

The cell culture were placed on standard slides and viewed under a light microscope. The morphological changes were documented with photomicrographs. A Nikon Microphot-SA microscope equipped with a UFX-DX metering system was used for all photomicrographs.

5.12. Detection of intracellular reactive oxygen species (ROS)

5.12.1. Preparation of DHE and H_2-DCF-DA stock solutions

DHE was dissolved in Me_2SO to the final concentration of 5 mg/ml. H_2-DCF-DA was dissolved in ethanol to the final concentration of 2.5 mg/ml. Stock solutions were kept in cold and dark.

5.12.2. Samples staining and preparation

- DHE staining - 10^9 cells of *R. glutinis* (control or treated by stress factors, See chapter 5.7.2.) were collected, washed three times with water and incubated with 100 µl of DHE stock solution for 10 min at room temperature, in the dark.
- H_2-DCF-DA staining - 10^9 cells of *R. glutinis* (control or treated by stress factors, See 5.7.2.) were collected, washed three times with water and incubated with 400 µl of H_2-DCF-DA stock solution for 1 hour at room temperature, in the dark.

After treatment with fluorescent dyes, cells were collected by centrifugation and washed with water. Around 2.5 µl of the concentrated cell suspension were spotted on a microscope cover slip coated with polylysine and laid on the microscope glass slide with mounting solution. Prepared microscope glasses were observed under the fluorescent microscope.

5.12.3. Fluorescence microscopy analysis

ROS production was vizualized by fluorescence microscopy using a Leica DMRXA2 fluorescence microscope. Emission of fluorescence due to oxidation of H_2-DCF-DA or DHE was detected by GFP or cy3 fluorescence filters, respectively. Digital images were captured with a coolSNAP camera driven by Openlab software. These images were then processed with Adobe Photoshop 6.0 to improve image quality.

5.13. DNA fragmentation determination

5.13.1. Protoplast preparation

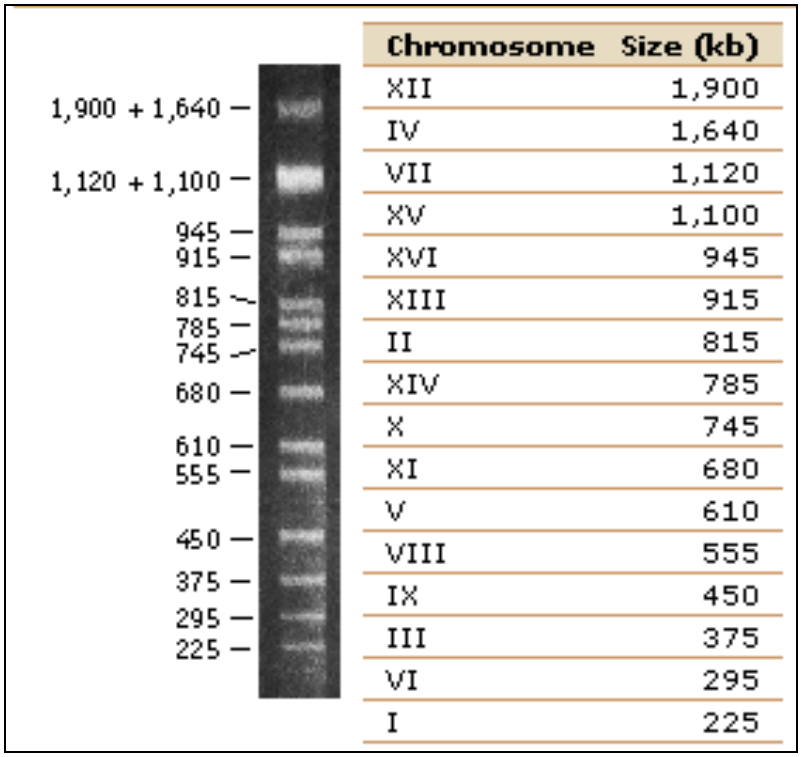
S. cerevisiae cells from 20 hour culture and *R. aurantiaca* and *S. shibatanus* cells from 48 hour cultures (See chapter 5.7.2.) were harvested by centrifugation (3500 rpm for 5 min.) and washed by osmotic stabilizer (1 M sorbitol). Pelet was resuspended in 300 µl of SCEM buffer (10 mM EDTA, 1 M sorbitol, 30 mM mercaptoethanol; 0.1 M sodium citrate, pH 5.8) and incubated with enzyme solution (10 mg/ml lyticase (320 U/mg) in 10 mM EDTA, 1 M sorbitol, 30 mM mercaptoethanol; 0.1 M sodium citrate, pH 5.8) at 24°C. Protoplast formation was observed under the light microscope.

5.13.2. Isolation of intact chromosomal DNA

S. cerevisiae cells from 24 hour culture and cells of carotenogenic yeasts from 75 hour cultures (See chapter 5.7.2.) were collected by centrifugation, washed with 50 mM EDTA pH 7.5 and suspended in enzyme solution (20 mg of lyticase/ml of 50 mM EDTA) to final concentration 4 mg of lyticase/1×10^9 cells. Suspension was then mixed with the same volume of 1% low melting point agarose in 125 mM EDTA pH 7.5 which was prewarmed to 42°C. This mixture was then incubated at 37°C for 30 min, distributed into molds and allowed to solidify at 4°C. The agarose plugs were then immersed in 5 ml of LET buffer (500 mM EDTA, 7.5% 2-mercaptoethanol, 10 mM Tris, pH 7.5) and incubated for 16 h at 37 °C. Plugs were immersed in 5 ml of NDS solution (2 mg/ml proteinase K in 500 mM EDTA, 1% lauroyl sarcosine and 10 mM Tris-HCl, pH 7.5) and incubated for 24 h at 50 °C. Incubation was repeated with 5 ml of freshly prepared NDS solution for 16 h at 50 °C. Plugs were then washed twice with 50 mM EDTA pH 7.5 and kept in this solution at 4 °C until use.

5.13.3. PFGE optimalization

Chromosomal DNA of model yeast *S. cerevisiae* and selected red yeasts was separated by contour-clamped homogeneous electric fields (CHEF) on a 15 cm square at 14 °C. The different electrophoretic conditions (agarose gel concentration of 1 – 1.2%; TBE molarity of 0.08 -1 M; voltage of 162 V, 135 V and 108 V; switch times of 70 s to 300 s) were applied to find optimal conditions for separation of chromosomes of red yeasts. Yeast Chromosome PFG Marker (16 chromosomes isolated from *Saccharomyces cerevisiae*, strain YPH80; size range: 225-1,900 kb; See chapter 5.3.) was used as standard for determination of size of separated chromosomes.



Chromosome	Size (kb)
XII	1,900
IV	1,640
VII	1,120
XV	1,100
XVI	945
XIII	915
II	815
XIV	785
X	745
XI	680
V	610
VIII	555
IX	450
III	375
VI	295
I	225

Figure 26: Yeast Chromosome PFG Marker

5.13.4. Gel staining and visualization

After electrophoresis, the gels were stained with 1 μg/ml of ethidium bromide for 1 hour and then destained for 30 min in destilled water. Gels were visualized under UV light and analyzed using SW Scionimage.

5.14. Gene knockout

5.14.1. Isolation of genomic DNA

Sz. pombe cells from 10 ml of overnight culture were harvested by centrifugation and resuspended in 0.5 ml of water. The pellet was resuspended in 0.2 ml of mixture (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0). Phenol:chloroform:isoamylalcohol (25:24:1) (0.2 ml) and acid-washed glass beads (0.4 ml) were added and the tube was vortexed for 5 min. After centrifugation for 5 min, the upper aqueous layer was transferred to a new tube, and 1/10 volume of 3 M NaOAc, pH 5.2 and 2.5 volume of 100 % ethanol were added. After mixing and centrifugation for 5 min, the pellet was washed with 70 % ethanol, spin, dried and dissolved in 400 μl of TE (pH 8.0). An aliquot of this DNA was then used as template for a PCR reaction.

5.14.2. PCR amplification of homology regions flanking the target gene

First the left and right homology regions 150 – 800 bp long, flanking the target gene (or up and down (dw) part of the target gene) were amplified by PCR. For one PCR reaction, a 1.1 × PCR premix was prepared with Dream Taq DNA Polymerase, 2 mM dNTPs, 1.1 × PCR buffer and template (genomic DNA) in 50 µl of total volume. 5 µl of up primers (5 µM upout and 5 µM upin) or dw primers (5 µM dwout and 5 µM dwin) were added into prechilled PCR tubes containing 50 µl of PCR premix. Tubes were then set into a PCR machine and the PCR was run as follows: 3 min 94°C; 30 cycles of 40 s 94°C, 50 s 60°C, 90 s 72°C; 5 min 72°C. Up and dw PCR products were then mixed together and purified over a Qiagen gel-extraction kit column. 10 µl of the PCR products with 2 µl of 6 × DNA loading buffer was run on a 1.5% agarose gel. The gel was compared with the gel preview at http://mendel.imp.ac.at /Pombe_deletion.

5.14.3. Insert preparation

Up and dw PCR products were firstly digested by Enzyme1 (Enz1): to the 52.2 µl of up and dw mixture were added 2 µl of restriction enzyme Enz1 and 5.8 µl of 10 × buffer and this was incubated 2 hours at 37°C. Digested products were then purified over a Qiagen gel-extraction kit column and ligated together: 52.2 µl of up and dw digested by Enz1 was mixed up with 2 µl of T4 DNA ligase and 5,8 µl of 10 × buffer and incubated 16 hours at 4°C. PCR primers are designed such that two homology regions are of unequal size; therefore, five constructs are expected after ligation. These correspond to the monomer of the smaller homology region, the monomer of the larger homology region, the homodimer of the smaller homology region, the desired heterodimer and the homodimer of the larger homology region. Resulting monomers and dimers were purified and finally digested by EnzA/EnzB: to the 52.2 µl of mix of dimers were added 1.5 µl of restriction enzyme EnzA, 1.5 µl of restriction enzyme EnzB and 5.8 µl of 10 × buffer and mix was incubated 2 hours at 37°C. After incubation, 60 µl of mix with 12 µl of 6 × DNA loading buffer was loaded on a 1 % agarose gel and run. The band corresponding to correctly ligated heterodimer of two homology regions was cut out using a scalpel according to the gel preview at http://mendel.imp.ac.at/Pombe_deletion. HD was extracted from gel slice using a a Qiagen gel-extraction kit column.

5.14.4. Vector preparation

The vector used for cloning of deletion constructs was pClonaKAN1. It contains the kanMX4 marker gene, conferring resistance to geneticine. This vector was digested by two restriction enzymes (EnzA/EnzB), run on agarose gel, cut out from gel, extracted from gel slice using a Qiagen gel-extraction kit column and dephosphorylated with shrimp alkaline phosphatase.

5.14.5. Cloning of homology regions into vector

Vector and inserts, both digested by EnzA/EnzB, were ligated together: 10 µl of extracted insert were ligated with approximately 10 ng of digested vector using T4 DNA ligase and incubated overnight at 4°C. Sample was mix up with 1.5 µl of T4 DNA ligase, 2 µl of 10 × buffer and up to 20 µl of H_2O.

5.14.6. Amplification of prepared plasmid in *E. coli*

Competent *E.coli* DH5α cells were used for transformation. 150 µl of competent cells were mixed with 10 µl of ligation mixture, incubated for 30 min on ice and subjected to heat shock for 90 s at 42 °C. The transformation mix was then transferred to 1 ml of prewarmed 2 × TY medium (10 g of yeast extract, 16 g of tryptone, 5 g of NaCl par 1 l of dH_2O) and incubated for 30 min at 37 °C with shaking. After incubation cells were plated on 2 × TY + ampicillin plates and icubated at 37 °C overnight. Positive colonies were detected by PCR followed by Enz1 digest.

5.14.7. Linearization of the plasmid and transformation into yeast.

Knockout plasmids were prepared from the positive *E.coli* transformants, digested by Enz1 and transformed into yeasts *Sz. pombe* sp286. The transformation was performed following lithium acetate method:

- Preparation of competent cells
  Sz. pombe cells were grown in YES medium to a density of approximately 1 × 107 cells/ml at 32°C. The cells were collected by centrifugation and the resulting pellet was washed three times with ice-cold sterilized water. This pellet was resuspended in 2 ml of ice-cold LiAc solution and incubated 30 min at 32°C.
- Transformation
  The competent cell suspension was mixed with 5–10 ng purified plasmid DNA and 145 µl polyethylene glycol (PEG) solution (50% PEG-4000), and then the mixture was vortex and incubated at 30 ◦C for 60 min. The cell suspension was heat-shocked at 43 ◦C for 15 min and allowed to cool at room temperature for 10 min. The cells were collected by a brief centrifugation (1600 × g, 3 min) and resuspended in 500 µl 1/2YE medium and incubated at 30 ◦C for 60 min while being shaken. The cell suspension was spread directly onto minimal selection plates. Transformant colonies appeared in 4–6 days at 30°C.

5.14.8. Verification of the knockout by colony-PCR

Gene knockouts were verified by colony PCR. Two independent colonies were randomly selected from a pool of recombinants and both were processed in parallel in subsequent analyses.

6. RESULTS AND DISCUSSION

6.1. Main characteristics of red yeast

In order to study yeast physiology under different conditions, it is important to know so called “reference parameters” which these yeasts possess under optimal condition. Red or carotenogenic yeasts are well known producers of valuable carotenoids. On agar plates they form characteristic yellow, orange and red coloured colonies. Red yeast can be of ellipsoidal or spherical shape (See *Figure 27*, *Figure 28*, *Figure 29*, *Figure 30*, *Figure 31* and *Figure 32*). Under optimal conditions (26°C, 100 rpm) they are able to grow up in 5 to 7 days. The growth curve of carotenogenic yeast is characterized by two-stepped course with long stationary phase (See *Graph 1*, *Graph 2*, *Graph 3*, *Graph 4*, *Graph 5* and *Graph 6*).

Characterisation of *Rhodotorula glutinis*

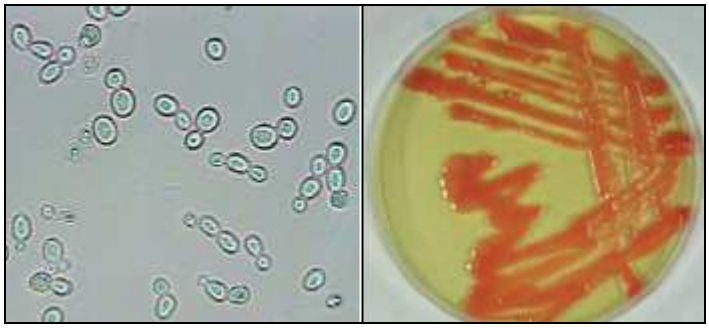
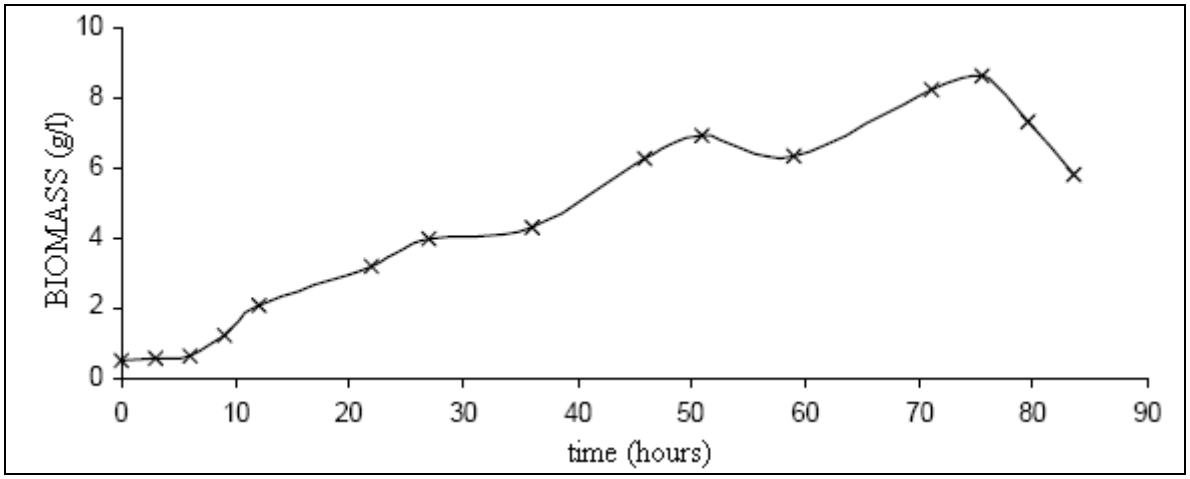


Figure 27: Microscopic image (640×) and streak plate of *Rhodotorula glutinis*



Graph 1: Growth curve of *Rhodotorula glutinis*

Characterisation of *Rhodotorula rubra*

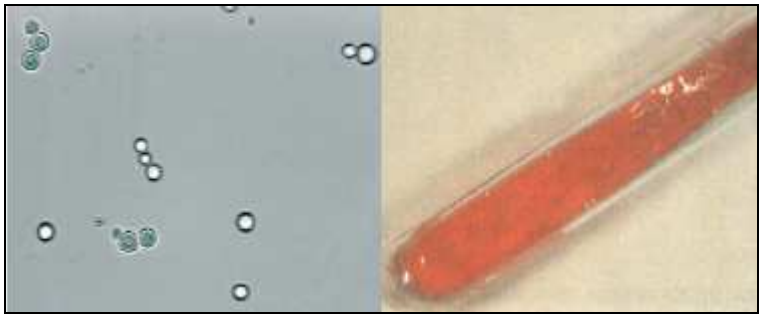
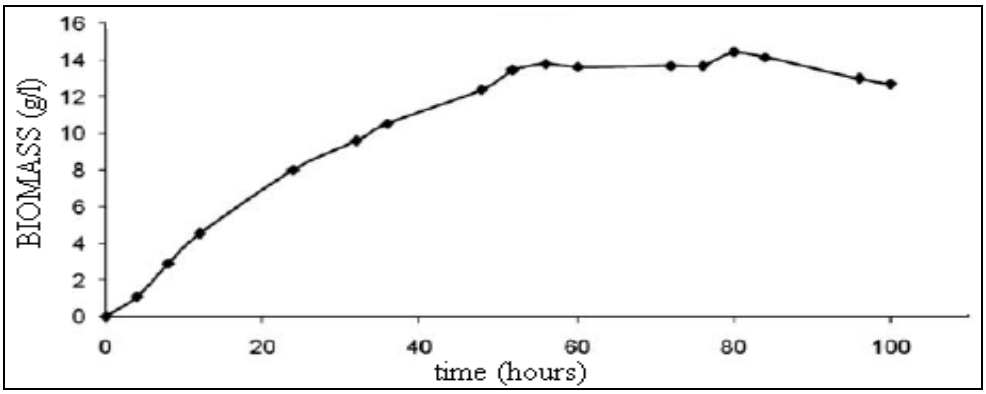


Figure 28: Microscopic image (640×) and agar slope of *Rhodotorula rubra*



Graph 2: Growth curve of *Rhodotorula rubra*

Characterisation of *Rhodotorula aurantiaca*

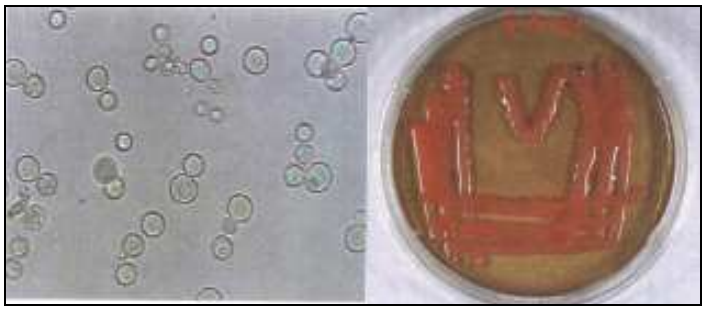
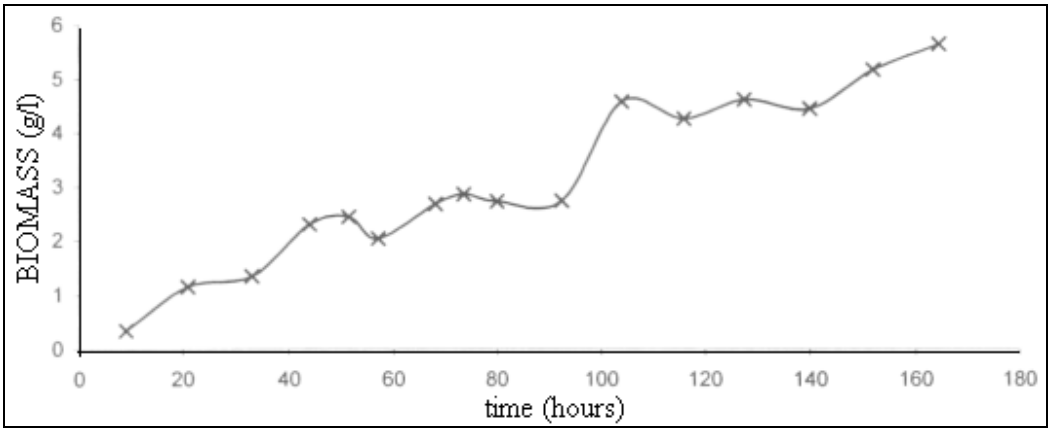


Figure 29: Microscopic image (640×) of *Rhodotorula aurantiaca*



Graph 3: Growth curve of *Rhodotorula aurantiaca*

Characterisation of *Sporobolomyces roseus*

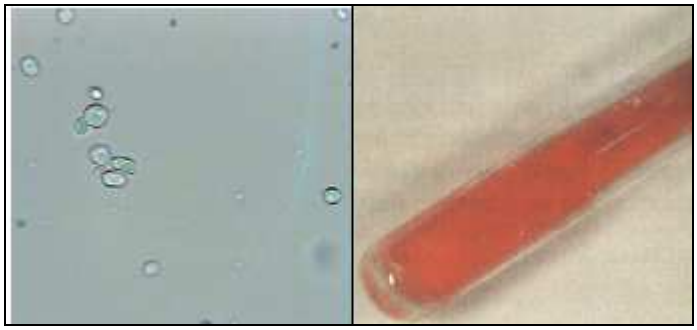
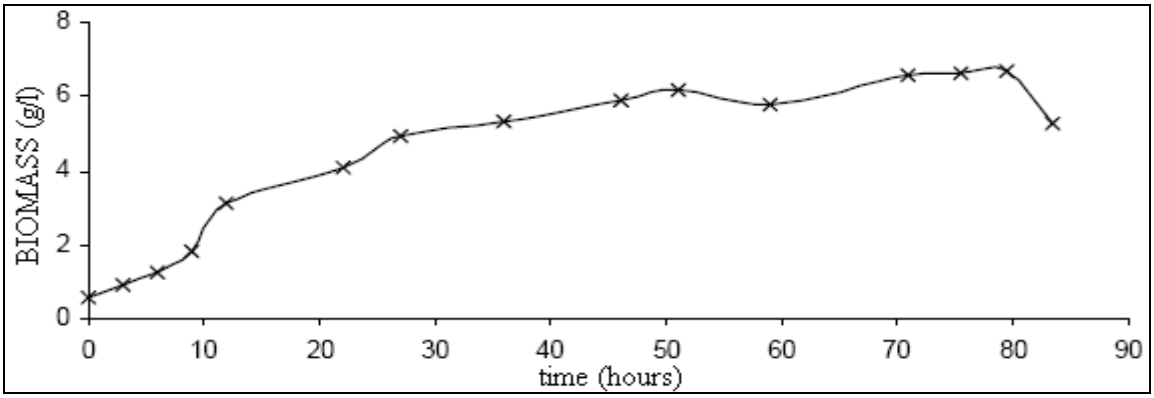


Figure 30: Microscopic image (640×) and agar slope of *Sporobolomyces roseus*



Graph 4: Growth curve of *Sporobolomyces roseus*

Characterisation of *Sporobolomyces shibatanus*

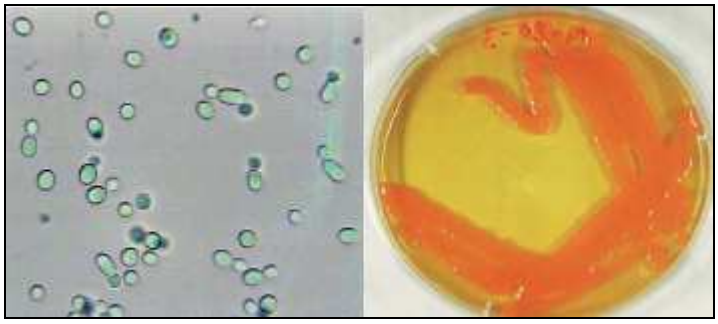
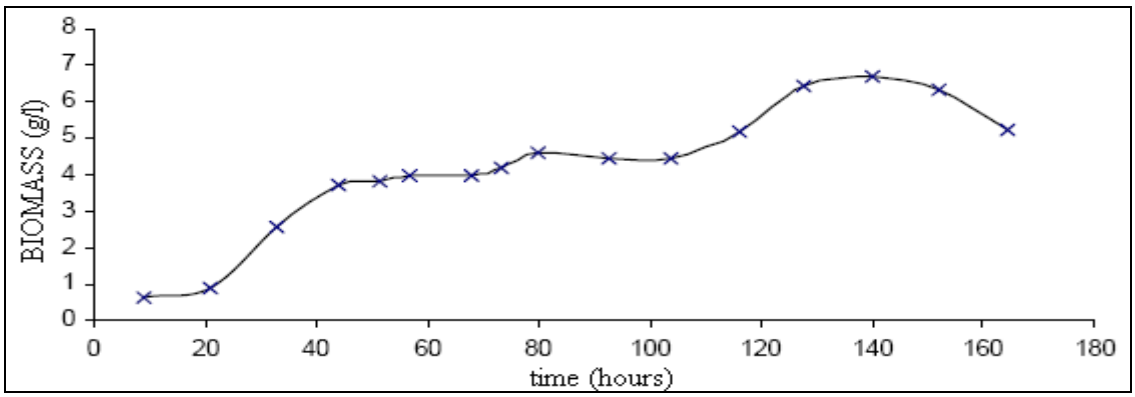


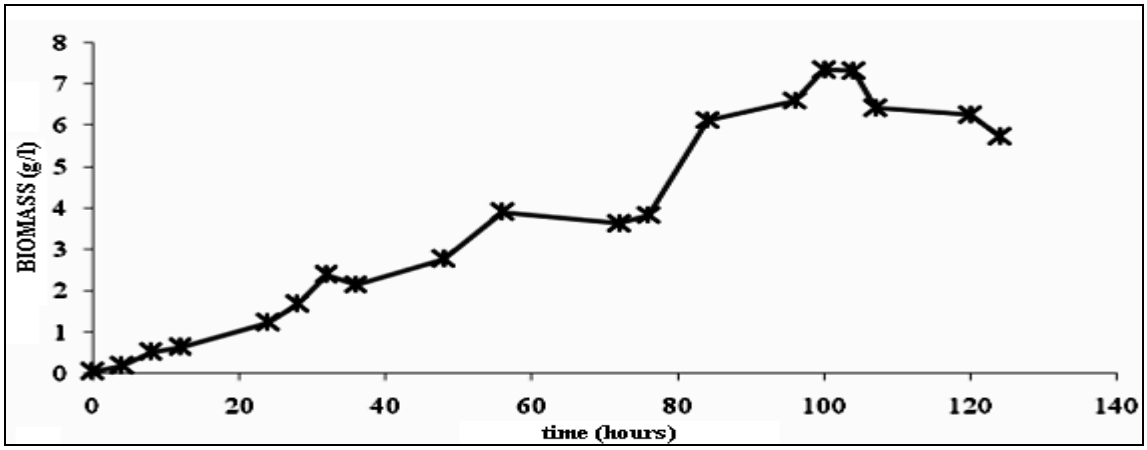
Figure 31: Microscopic image (640×) and streak plate of *Sporobolomyces shibatanus*



Graph 5: Growth curve of *Sporobolomyces shibatanus*

Characterisation of Cystofilobasidium capitatum

Figure 32: Microscopic image (1000×) and streak plate of *Cystofilobasidium capitatum*



Graph 6: Growth curve of *Cystofilobasidium capitatum*

Comparison of presented growth curves led to some partial conclusions about growth of red yeasts. All tested strains reached stationary phase after about 50 hours of cultivation. All strains also exhibited prolonged stationary phase with at minimum one, more often with several growth maxima. First growth maximum was observed in all strains after about 80 hours of growth. In strains followed for longer time than 100 hours additional growth maximum was observed after 105 – 140 hours. Carotenogenic yeasts probably utilize some endogenous substrates accumulated at the beginning of stationary phase. Growth maxima are mostly accompanied with carotenoid production maxima (See chapter 6.3.4.1., *Graph 10*; chapter 6.3.5.1., *Graph 15* and chapter 6.3.6.1., *Graph 17*), mainly in first 90 hours of cultivation. For all stress and waste experiments cultivation in production media was carried out for 80 hours (to first production maximum) to eliminate potential growth inhibiton caused by nutrient starvation or toxic effect of stress. Longer cultivation can be also complicated by higher ratio of dead and living cells and in semi-large-scale and large-scale experiments also with higher production costs.

6.2. Growth of red yeasts on various waste substrates

Growth of red yeasts is strongly influenced by cultivation conditions (pH, temperature, aeration...). To assess the growth of yeast on various carbon and nitrogen sources, the same cultivation conditions have to be ensured. Temperature of 26°C and aeration of about 100 rpm have been reported as optimal cultivation conditions for growth of red yeast. The total biomass yield was studied in a culture medium comprising glucose (40 g/l) as carbon source and yeast extract (7 g/l) as nitrogen source. The growth of red yeast in culture medium described above was compared with the growht of red yeast on diverse waste substrates, such as whey, potato mass, apple mass or various cereals to examine the potential of these materials as substrates for red yeast cultivation (See chapter 5.7.5., *Table 12*, *Table 13*, *Table 14* and *Table 15*)

6.2.1. Growth of *Rhodotorula glutinis* cultivated on various waste substrates

Table 16, *Table 17*, *Table 18* and *Table 19* summarize results of *R.glutinis* biomass production on liquid or processed whey (wt – conventional glucose medium; WL – whey lyophilized; WLA – whey lyophilized, acidified; Wl – whey liquid; PE – potato extract; 1/2/3 – number of series), potato mass (wt/p - conventional glucose medium in potato experiment; G-PE – glucose + potato extract; PE – potato extract; G-PP – glucose + potato peels; PP – potato peels; 1/2 – number of series), apple mass (wt/a - conventional glucose medium in apple experiment; AF – apple fiber; G-AF – glucose + apple fiber; CA – crushed apple; AP – apple peels; 1/2 – number of series) and several cereal based substrates (wt/c - conventional glucose medium in cereal experiment; meal – wheatmeal; pastes – pastes; AF – apple fiber; WGF – whole-grain frumenty; grains – grains; bran – wheatbran). Growth parameters are compared in *Graph 7*.

Table 16: Growth of *R. glutinis* cultivated on whey and potato extract

	wt 1	WL1	WLA 1	Wl 1	PE 1	wt 2	WL 2	WLA 2	Wl 2	wt 3	PE 3
CDW (g/l)	8.96	5.84	9.05	4.47	7.26	7.03	8.13	8.12	4.52	7.66	5.51

Table 17: Growth of *R. glutinis* cultivated on potato mass

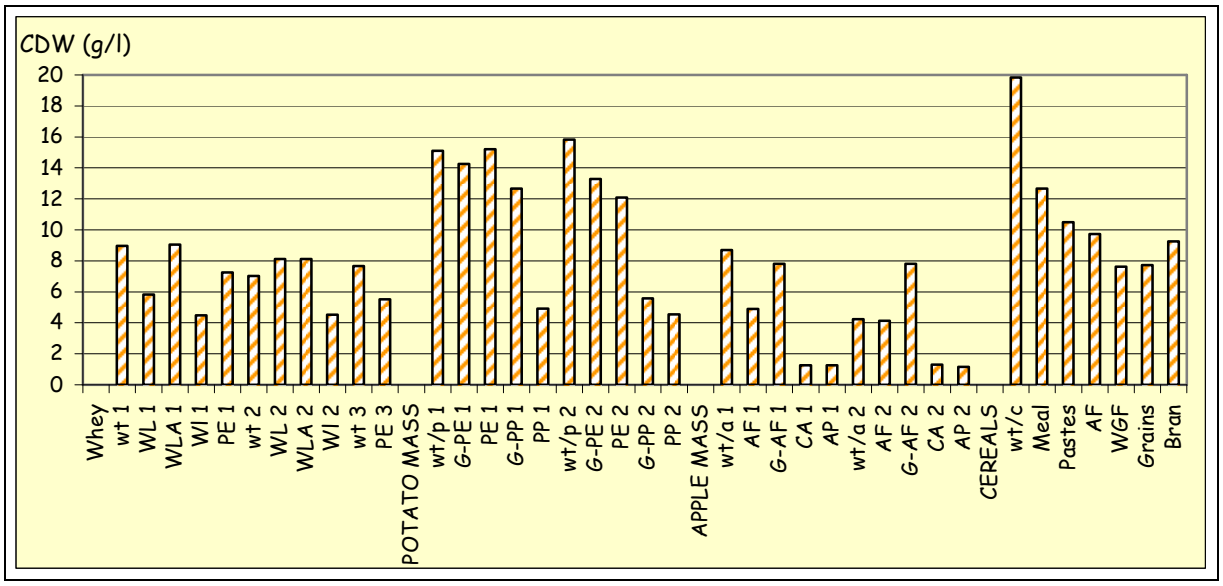
	wt/p 1	G-PE 1	PE 1	G-PP 1	PP 1	wt/p 2	G-PE 2	PE 2	G-PP 2	PP 2
CDW (g/l)	15.12	14.24	15.22	12.65	4.90	15.84	13.27	12.09	5.56	4.54

Table 18: Growth of *R. glutinis* cultivated on apple mass

	wt/a 1	AF 1	G-AF 1	CA 1	AP 1	wt/a 2	AF 2	G-AF 2	CA 2	AP 2
CDW (g/l)	8.70	4.90	7.82	1.25	1.25	4.23	4.13	7.82	1.30	1.15

Table 19: Growth of *R. glutinis* cultivated on cereal-based substrates

	wt/c	Meal	Pastes	AF	WGF	Grains	Bran
CDW (g/l)	19.84	12.65	10.50	9.72	7.62	7.72	9.26



Graph 7: *R. glutinis* growth on various waste substrates

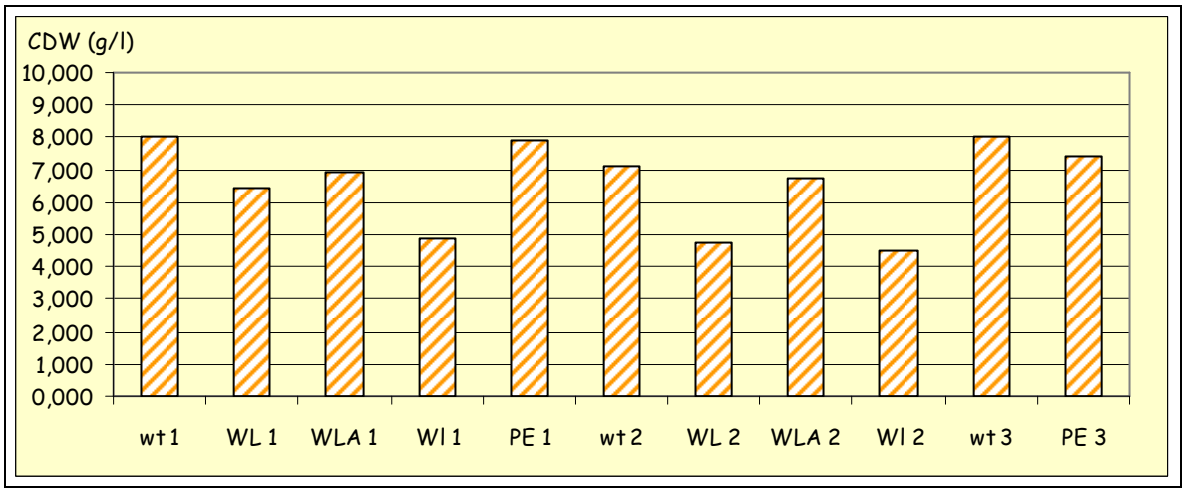
Cultivation of red yeast *Rhodotorula glutinis* can be performed in medium with whey or potato mass as carbon source. Growth of *Rhodotorula glutinis* on lyophillized, deproteined whey added into both inoculum II and production media or only into production medium is comparable with its growth in optimal glucose medium. Also potato mass can be used as carbon source. The best biomass yields were observed for cultivation in medium with potato extract as the only carbon source or potato extract with glucose present either in inoculum II and production media or only in production media. *Rhodotorula glutinis* wasn't able to assimilate apple fibre, unless glucose wasn't present in medium. Using of some cereal materials as carbon source reduced biomass production by half (See *Graph 7*).

6.2.2. Growth of *Rhodotorula rubra* cultivated on various waste substrates

In *Table 20* and *Graph 8*, results of *R. rubra* biomass production on liquid or processed whey and potato mass (for sample descriptions please see chapter 6.2.1.) are summarized.

Table 20: Growth of *R. rubra* cultivated on whey and potato mass

	wt 1	WL1	WLA 1	Wl 1	PE 1	wt 2	WL 2	WLA 2	Wl 2	wt 3	PE 3
CDW (g/l)	8.02	6.44	6.89	4.87	7.88	7.13	4.74	6.74	4.49	8.04	7.41



Graph 8: Growth of *R. rubra* cultivated on whey and potato extract

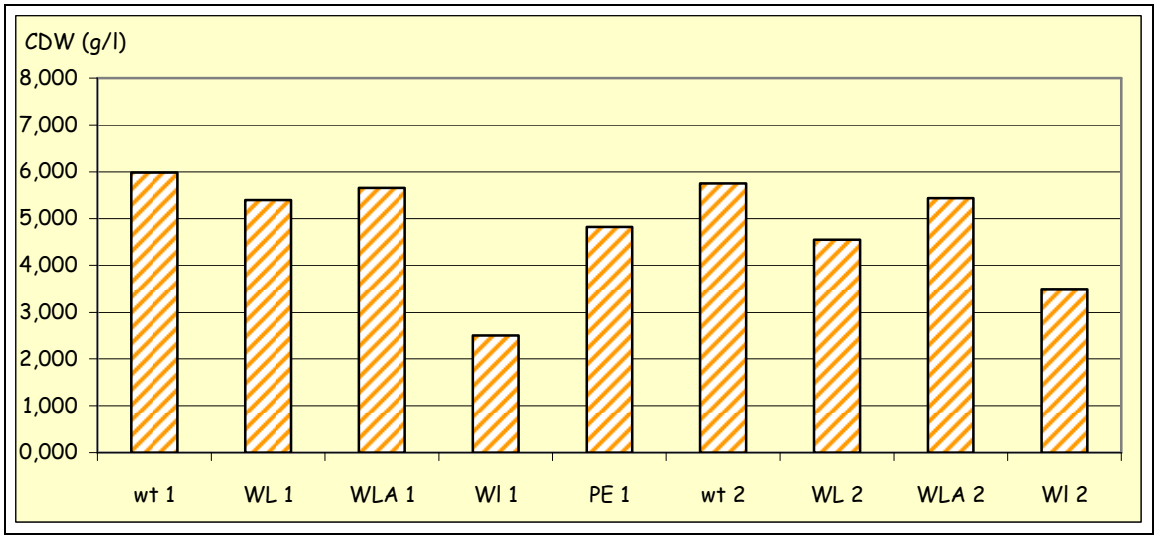
Red yeast *Rhodotorula rubra* is able to use both whey and potato extract as carbon source. The best results were obtained for lyophillized, deproteined whey and potato extract added either only into production media or into inoculation II and production (See *Graph 8*).

6.2.3. Growth of *Sporobolomyces roseus* cultivated on various waste substrates

In *Table 21,* results of *S. roseus* biomass production on liquid or processed whey and potato mass (for sample descriptions please see chapter 6.2.1.) are presented.

Table 21: Growth of *S. roseus* cultivated on whey and potato mass

	wt 1	WL1	WLA 1	Wl 1	PE 1	wt 2	WL 2	WLA 2	Wl 2
CDW (g/l)	5.98	5.39	5.65	2.51	4.82	5.75	4.55	5.43	3.49



Graph 9: Growth of *S. roseus* cultivated on whey and potato extract

In the case of *Sporobolomyces roseus* all tested materials can be used for cultivation, except for non-processed liquid whey, where the biomass production was lower by half than in wild type (See *Graph 9*).

In this part of experimental work, the potential of several waste materials (whey, potato mass, apple mass and various cereals) as substrates for biomass production by some yeast strains belonging to the genus *Rhodotorula* and *Sporobolomyces* were examined. Our results demonstrated that potato extract or lyophillized whey non-processed as well as deproteinated could be used as a suitable carbohydrate source for red yeast basic metabolism. Further, carotenoid and ergosterol production by red yeast cells growing on these waste substrates were measured.

6.3. Carotenoid production by red yeast cultivated on various waste materials

Red yeasts produce the major carotenoid pigments, namely yellow β-carotene and orange-red ketocarotenoids, such as torularhodin and torulene. The type, concentration and yield of various carotenoids depend on the species of microorganism and their culture conditions. Agro-industrial by-products and surpluses that frequently create serious environmental problems may be possibly used as low-cost nutrient sources for microbial fermentations. In this work, the potential of several waste materials (whey, potato mass, apple mass and various cereals) as substrates for the production of carotenoids by some yeast strains belonging to the genus *Rhodotorula* and *Sporobolomyces* were examined.

To assess carotenoid production by red yeast, they need to be first extracted from the cell, separeted from each other and identified. However the carotenoid extraction and analysis can often represent a serious problem because of thermal labilty of carotenoid pigments and their sensitivity to the light (See chapter 3.4.1.2.). Moreover, many of carotenoids are structurally closely related and so resolution of their separation is often unsatisfying. Carotenoid sample preparation and analysis require quick work with temperature and light effect minimizing. In recent years, PI electrospray mass spectrometry is widely used for individual carotenoid identification (See chapter 3.4.1.2.).

6.3.1. Visual assesment of carotenoid production

The rough profile of carotenoids produced by yeast can be estimated by visual observation of culture colour.

- cultivation under oxidative and osmotic stress

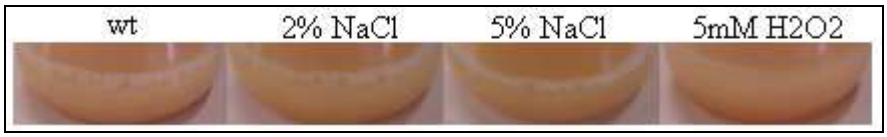


Figure 33: *R. glutinis* cultivation under osmotic and oxidative stress

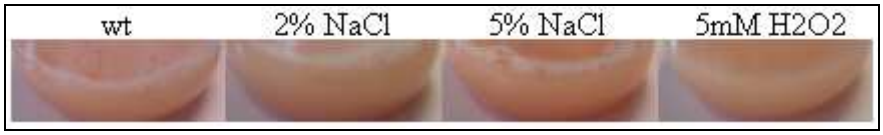


Figure 34: *R. rubra* cultivation under osmotic and oxidative stress

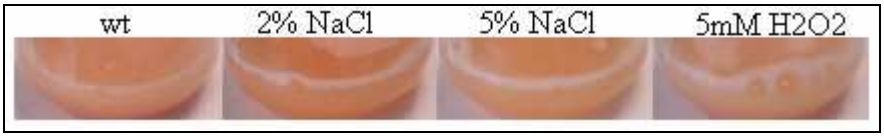


Figure 35: S. roseus cultivation under osmotic and oxidative stress

Colour of culture in media with osmotic (2 % NaCl and 5 % NaCl) or oxidative (2 mM H_2O_2 and 5 mM H_2O_2) stress wasn't markedly different from colour of culture in glucose media. Conventional orange-red colour of yeast was observed in all mentioned media (See *Figure 33*, *Figure 34* and *Figure 35*). When higher concentrations of osmotic or oxidative stress were used the culture colour was altered. This alteration can suggested changes in quantity and/or quality of produced carotenoids.

- cultivation on whey and potato mass

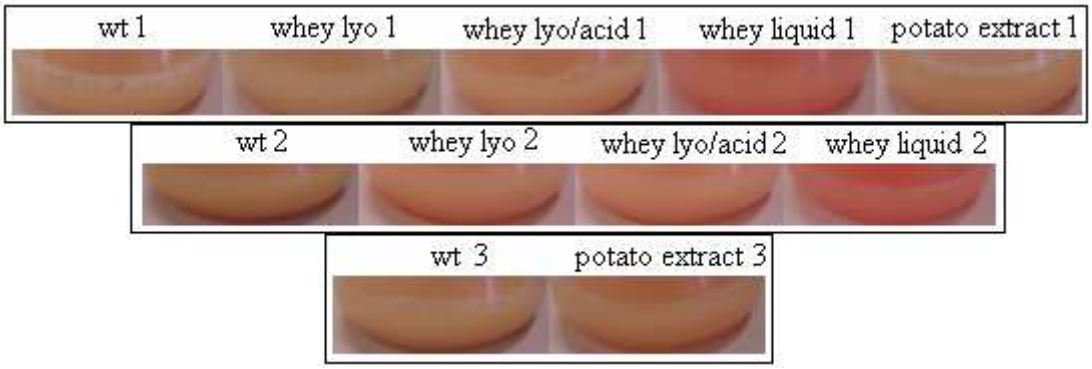


Figure 36: *R. glutinis* cultivation on whey and potato ectract

Cultivation of *Rhodotorula glutinis* on different forms of whey and potato extract didn't lead to alteration of culture colour except for cultivation in liquid whey. When cultivation was performed on liquid whey pink coloured culture was obtained. Therefore changes in profile of produced carotenoids were expected, but they weren't confirmed by HPLC analysis.

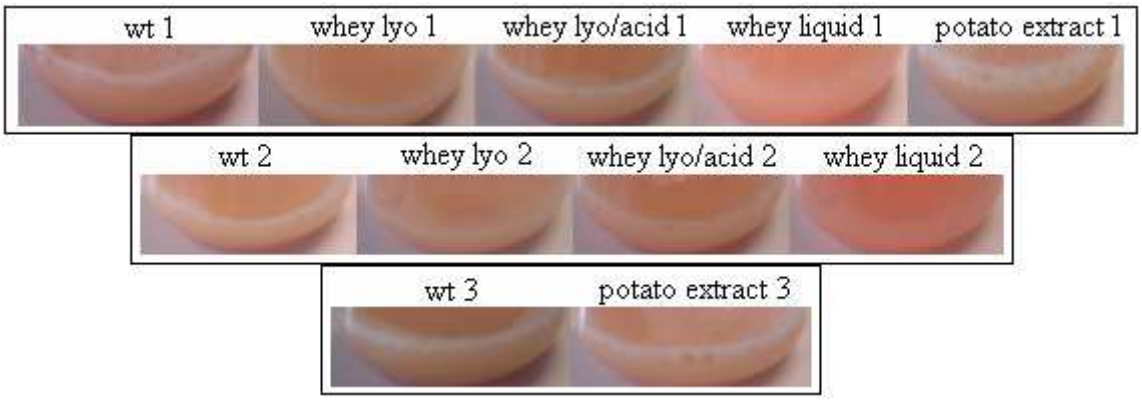


Figure 37: *R. rubra* cultivation on whey and potato extract

For majority of waste materials used for *Rhodotorula rubra* cultivation orange red coloured cultures were reached. Just as in the case of *Rhodotorula glutinis* some differences were observed only for cultivation in liquid whey (See *Figure 37*). The colour of this culture was brighter than other cultures what suggests weaker growth of yeast in this type of media. It was confirmed also by cell density measurements (See chapter 6.2.2.).

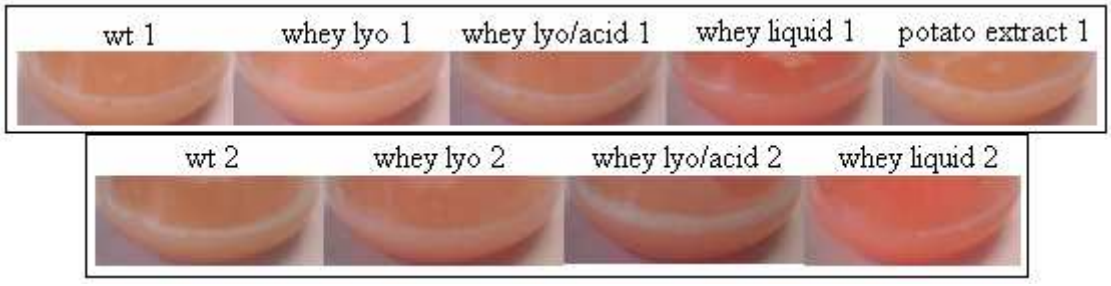


Figure 38: *S. roseus* cultivation on whey and potato extract

Also for *Sporobolomyces roseus* the most important differences of culture colour were observed in media with liquid whey (See *Figure 38*). It seems that liquid whey represents an activator for a pink carotenoid formation. This conclusion couldn't be confirmed because of weak growth of red yeast on liquid whey.

- cultivation on potato mass

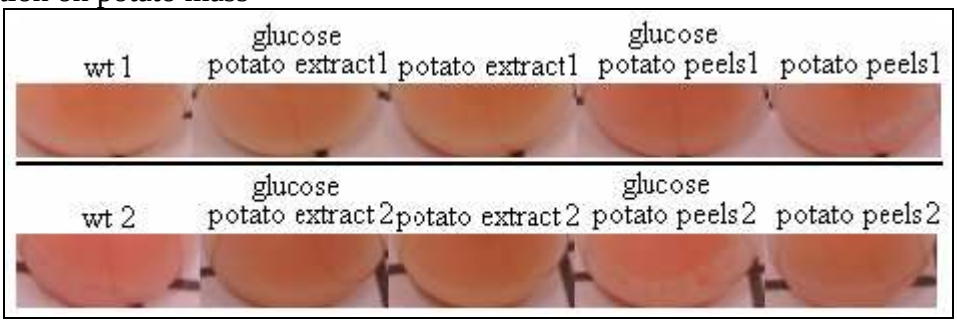


Figure 39: *R. glutinis* cultivation on potato mass

Colour of *Rhodotorula glutinis* in media with potato extract was similar as in glucose media. If potato peels were used for yeast cultivation more red cultures were obtained. Therefore slightly higher production of torularhodin and torulene was expected.

- cultivation on apple mass

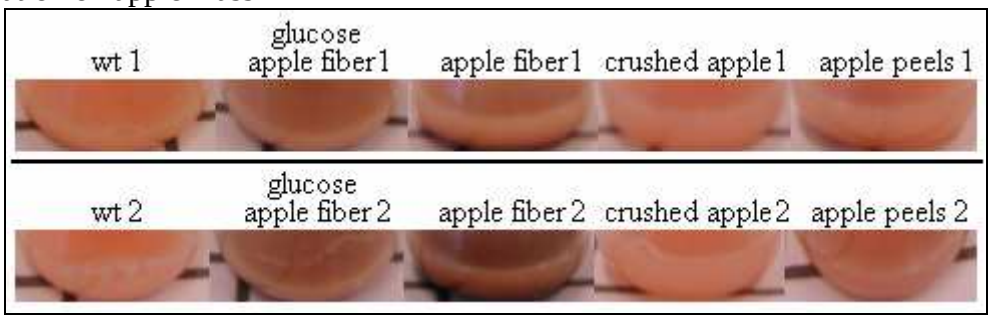


Figure 40: *R. glutinis* cultivation on apple mass

If apple fibre or apple mass were comprised in media the estimation of carotenoid production according to culture colour couldn't be performed because of more intensive red-brown colour of this material.

- cultivation on various cereal-based substrates

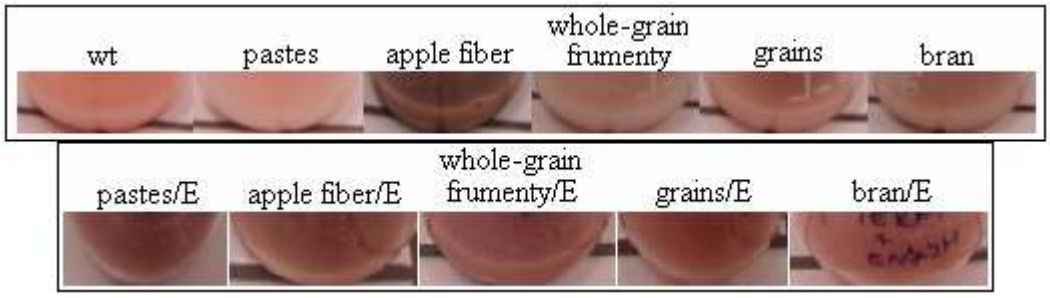


Figure 41: *R. glutinis* cultivation on various cereal-based substrates

Presence of diverse coloured cereal-based materials in media used for *Rhodotorula glutinis* cultivation distorted perception of colour of yeast. Carotenoid production therefore couldn't be estimated by sight.

6.3.2. Separation and identification of carotenoids by RP-HPLC/PDA-ESI-MS

6.3.2.1. Carotenoid separation by HPLC

Accurate identification and quantitation of all analytes present in a sample requires their complete chromatographic resolution. Separation of complex mixtures of carotenoids, many of which are closely related structurally, can often represent serious problem. High-performance liquid chromatography (HPLC) is regarded as the preferred method for the separation of carotenoids found in biological matrices. In order to get the highest possible resolution, separation conditions need to be first optimized. Satisfactory separation of carotenoid pigments extracted from red yeast can be carried out using a C18 reversed-phase column and methanol as the solvent system at a flow rate of 1 ml/min (See *Figure 42*).

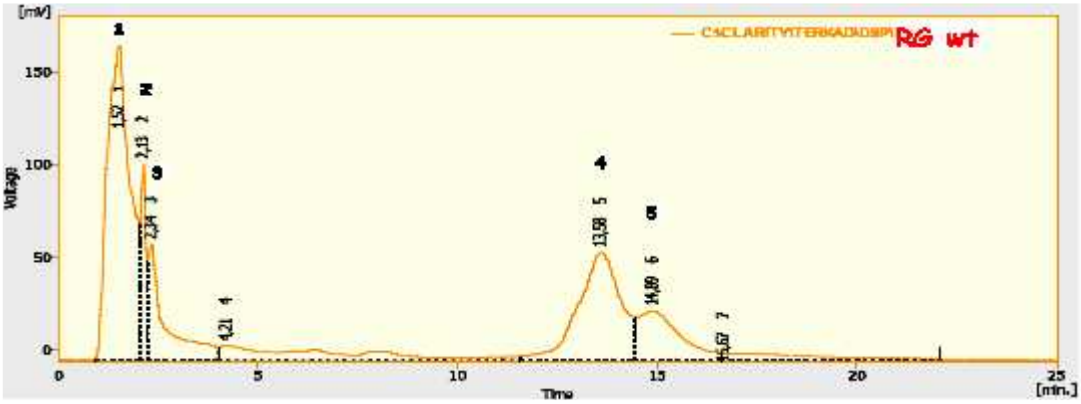


Figure 42: HPLC chromatograme of carotenoids from *Rhodotorula glutinis*

6.3.2.2. Identification of carotenoids by absorbance spectra

The advent of photodiode array detection, allowing for continuous collection of spectral data during HPLC analysis, has provided a powerful tool for research. Absorption spectra of separated analytes can be recorded and compared with UV/VIS spectra and retention times of authentic standards. The similarities in absorbance spectra of individual carotenoids (See *Figure 22* and *Figure 43*) complicate carotenoid identification by this technique.

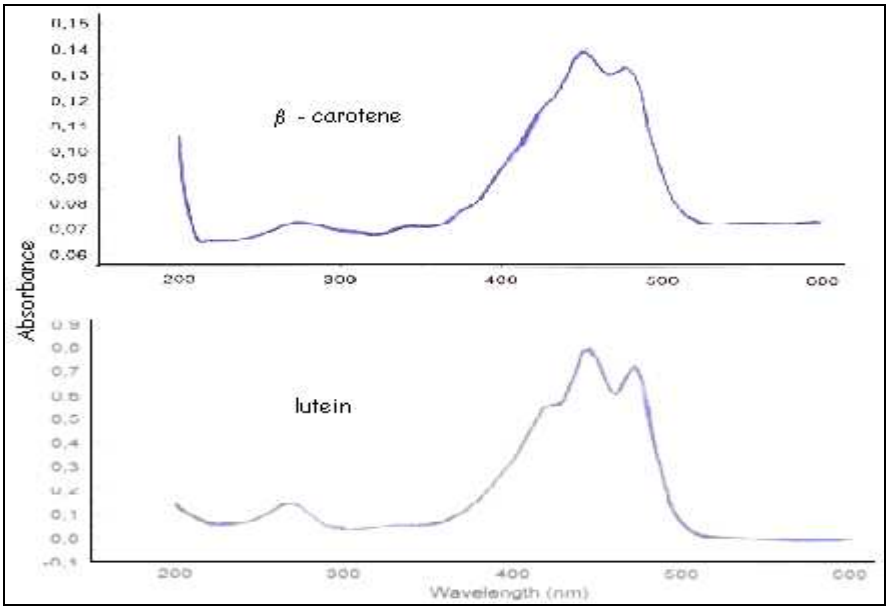


Figure 43: Absorption spectra of carotenoid standards

6.3.2.3. Identification of carotenoids by mass spectra

Mass spectrometric and tandem mass spectrometric analyses, which provide molecular weight and characteristic fragmentation patterns, may provide final confirmation of individual carotenoids when used in conjunction with retention and spectral characteristics. Several ionization methods have been reported for mass spectrometric analysis of carotenoids. In the last years, positive ion electrospray LC-MS was shown to be useful for the analysis of carotenes. However, because carotenes lack any heteroatom such as oxygen to which protons or sodium cations might attach, no ions can be formed by ESI. In order to ionize carotenes such as β-carotene during electrospray LC-MS, solution-phase oxidation of carotenoids has to be carried out by means of postcolumn addition of a halogenated solvent (for example chloroform) to the HPLC effluent.

In order to develop an electrospray LC-MS method for carotenoids, an analysis of β-carotene standard was carried out to determine suitable electrospray parameters. Ammonia was added to the standard solution. Under these conditions, molecular ion of β-carotene, $M^{\cdot+}$, was observed at m/z = 536 (See *Figure 44*). Peaks at m/z = 288 and 316 in mass spectrum of β-carotene standard were identified as phtalates which have been supposed to origin from plastic ware used for carotenoid preparation.

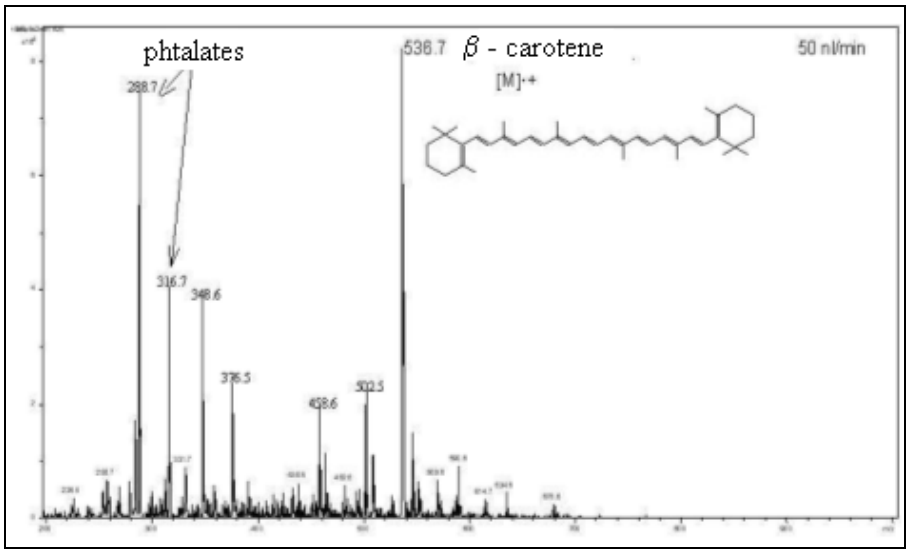


Figure 44: Mass spectrum of β-carotene standard

With the view to minimize the effect of phtalates, new carotenoid analysis was carried out in glass ware and prepared sample was analyzed as follow: ammonia was added to the sample and it was loaded by ES needle with 25 µm of diameter and 40 cm of length; at flow of 60 nl/min and at 4000 V. Under these conditions, no peaks at m/z = 288 and 316 were observed (See *Figure 45*). It suggets that glass ware can avoid formation of undesirable ions.

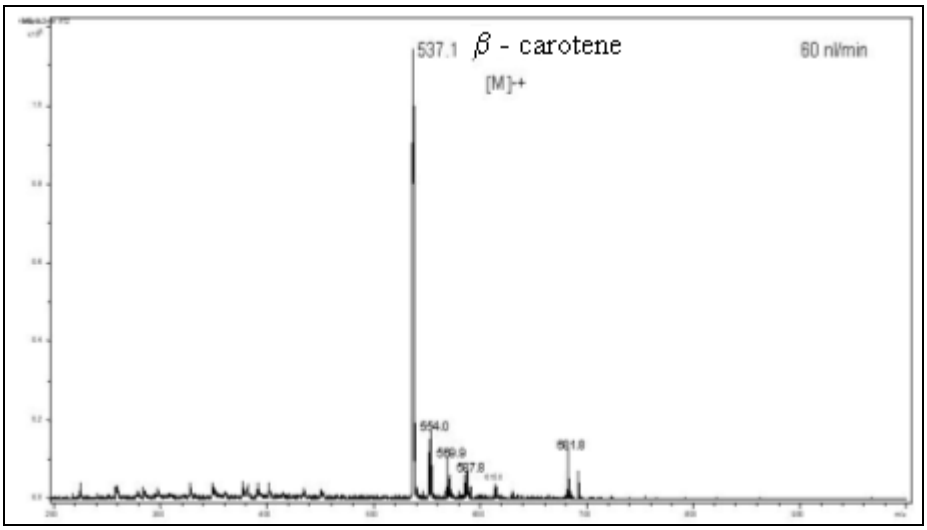


Figure 45: Mass spectrum of β-carotene standard after carotenoid preparation optimalization

MS spectrum of *RG* extract (See *Figure 46*) confirmed the presence of *β*-carotene as the major carotenoid in carotenogenic yeasts. However, LC-MS analysis of carotenoid mixtures n biological exteact is prety impossible due to carotenoid properties, such as thermic and light instability and need further optimization.

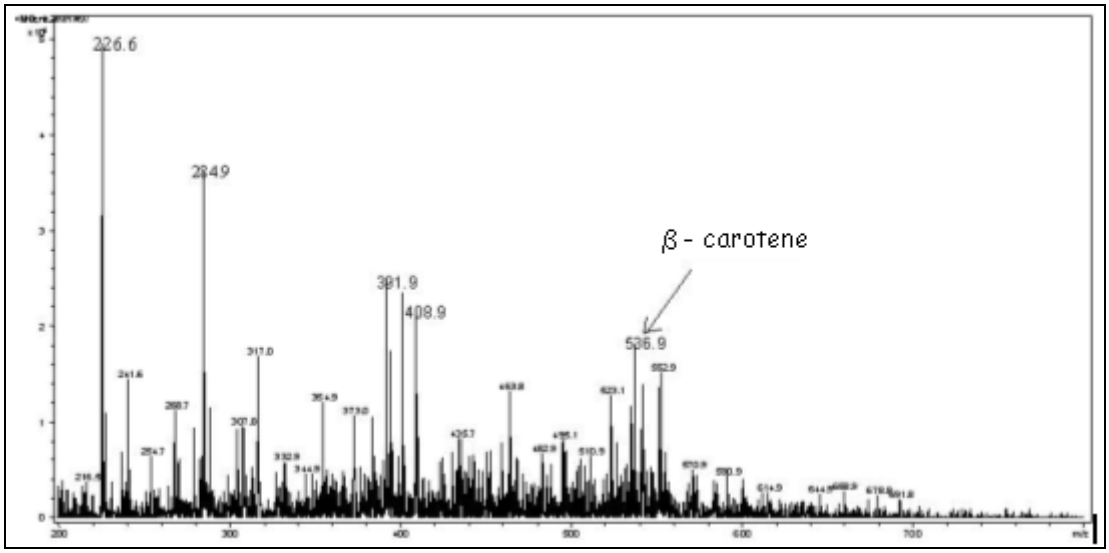


Figure 46: Mass spectrum of *R. glutinis* extract

Based on all discussed facts it can be concluded that a typical HPLC chromatogram of carotenoids present in the *Rhodotorula glutinis* extract after 80-hour cultivation in production medium exhibits 5 dominant peaks (See *Figure 47*). By detailed LC/PDA/MS analysis of carotenoid pigments, described in Materials and Methods (See 5.10.), following carotenoids: peak **1** (3.81 min) – torularhodin alcohol (absorption maxima 419, 452, 496 nm; m/z 552.9) ; peak **2** (4.96 min) – torularhodin (absorption maxima 425, 453, 486 nm; m/z 565.97); peak **3** (7.25 min) – torulene (absorption maxima 453, 489, 518 nm; m/z 534.38); peak **4** (12.79 min) – lycopene (absorption maxima 438, 469, 518 nm; m/z 536,63) and peak **5** – β,β-carotene (absorption maxima 425, 450, 483 nm; m/z 536.46) were identified.

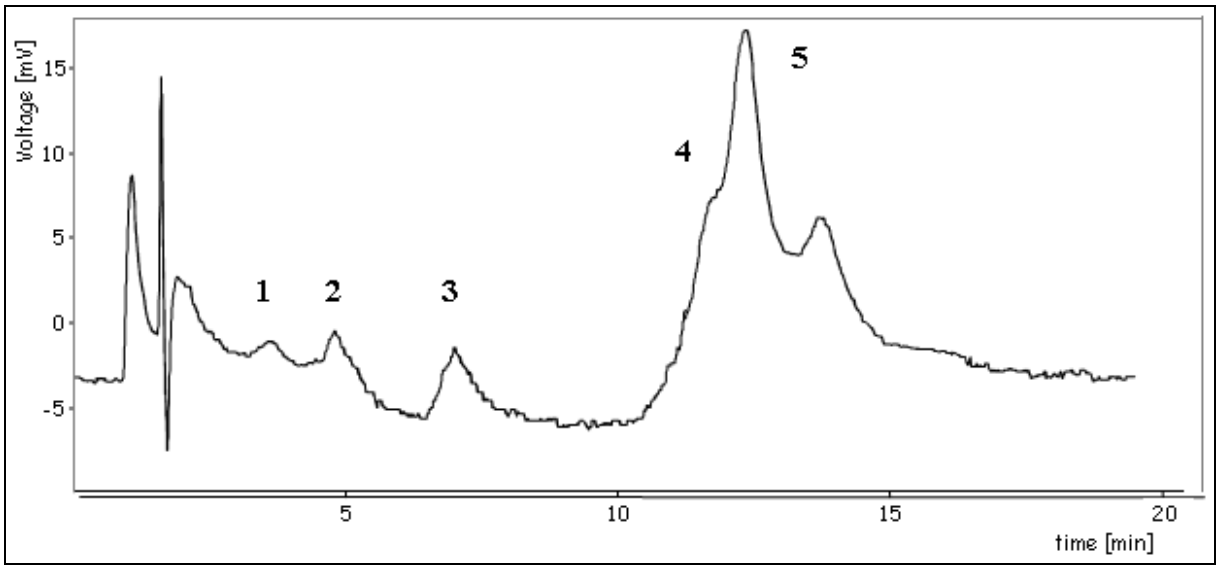


Figure 47: A typical HPLC chromatogram of carotenoids present in the *Rhodotorula glutinis* extract after 80-hour cultivation in production medium

Advantages of combined HPLC/PDA/MS-ESI technique for carotenoids include lower limits of detection, abundant molecular ions of both xanthophylls and carotenes, substantially improved ease of operation, reduced maintenance and higher sample throughput. Use of autosampler was excluded with regard to carotenoid unstability. For carotenoid quantitation available standards were used. Based on HPLC chromatographic data, total carotenoid production could be approximately expressed as about 1.5-fold amount of beta-carotene (See chapter 3.4.1.2.).

6.3.3. Determination of carotenoid production by RP-HPLC/UV-VIS

In the current literature, yeasts *Rhodotorula* are described as a potential source of carotenoids with medical or industrial interest. β-carotene was identified as the major carotenoid pigment of these red yeasts, mainly in experiments carried out for 80 hours of cultivation in production media (See chapter 6.3.2.3.). According to experimental data it seems that during 80-hour cultivation of *R.glutinis* cells carotenogenic biosynthetic pathway is not finished because of presence of lycopene (direct biosynthetic precursor of β-carotene) and relative low amount of torulene and torularhodin (final oxidized products of carotenoid pathway in *Rhodotorula* cells) found in yeast cell extract.

β-carotene production by yeast can become industrially feasible if the cost of production can be minimized. The utility of different waste substrates as a nutrient source for the production of β-carotene by red yeasts *Rhodotorula* and *Sporobolomyces* was tested. β-carotene content was measured by HPLC with spectrophotometric detection and quantified against peak area calibrations obtained from standard curve:

$$A[mV.s] = 14.237 \times c_{\beta\text{-carotene}}\ [\mu g/ml]$$

6.3.4. Carotenoid production by red yeast *Rhodotorula glutinis*

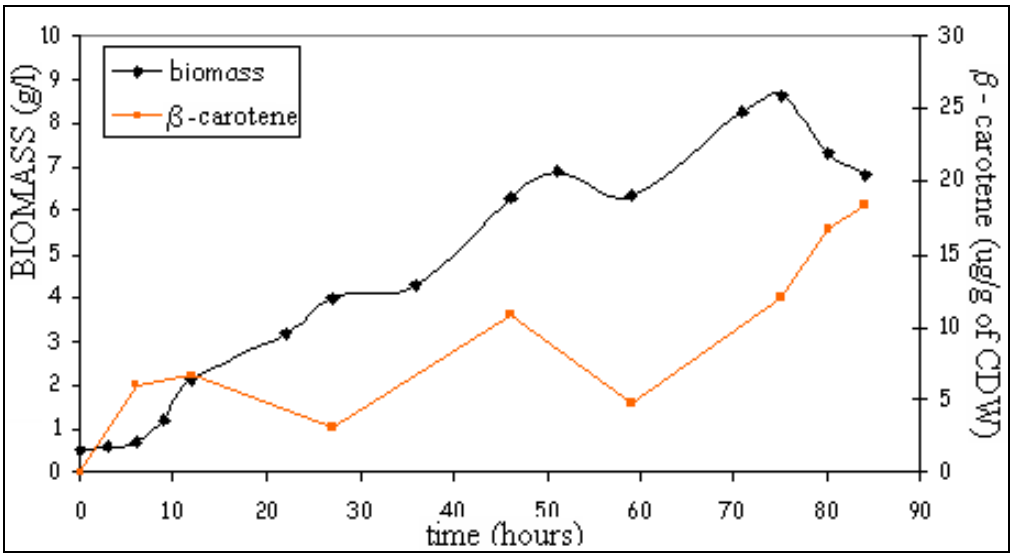
In next chapters 6.3.4.1. and 6.3.4.2., biomass and β - carotene production by red yeast *Rhodotorula glutinis* are discussed.

6.3.4.1. Carotenoid production by *Rhodotorula glutinis* cultivated in optimal conditions

In *Table 22*, biomass and β - carotene production by *R. glutinis* are summarized. Similarities of growth curve and carotene-production course are illustrated in *Graph 10*.

Table 22: Biomass and carotenoid production by *Rhodotorula glutinis*

cultivation time (hours)	biomass (g/l)	β-carotene (μg/g CDW)
0	0.50	0
3	0.61	-
6	0.66	6.04
9	1.21	-
12	2.11	6.62
22	3.18	-
27	4.01	3.10
36	4.30	-
46	6.28	10.90
51	6.91	-
59	6.36	4.75
71	8.26	-
75	8.62	12.05
80	7.31	16.78
84	5.82	18.37



Graph 10: Carotenoid production by *Rhodotorula glutinis*

Maximal carotenoid production by *Rhodotorula glutinis* cultivated in optimal medium was observed in 84th hour of cultivation (18.37 μg/g cell dry weight).

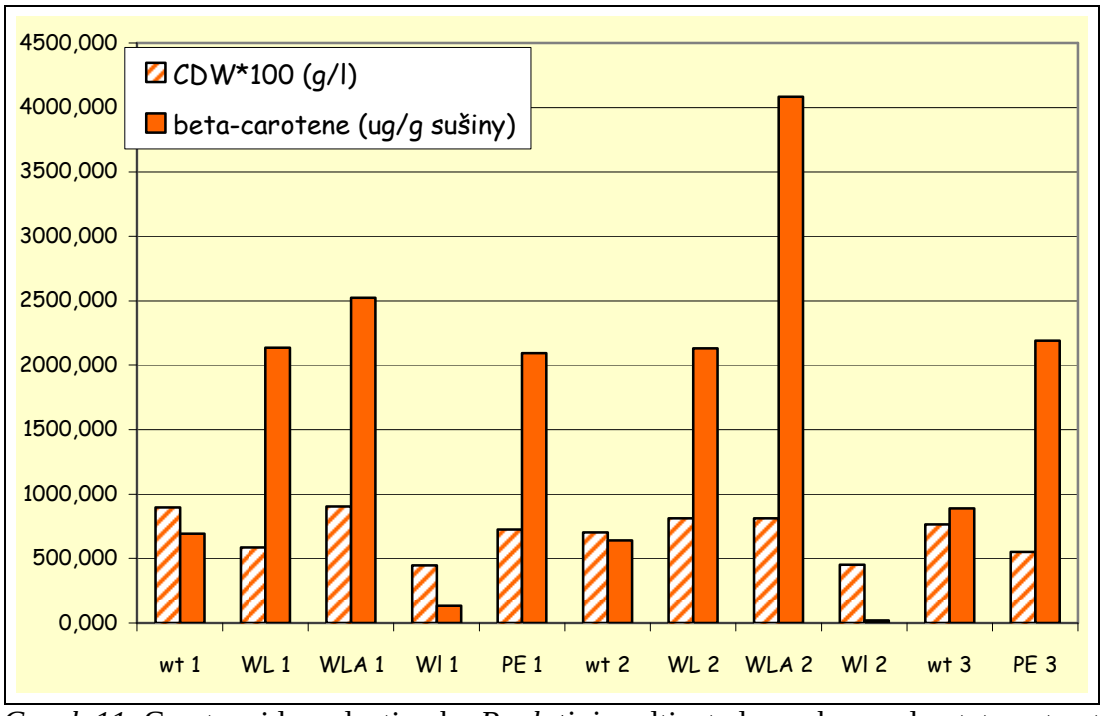
6.3.4.2. Carotenoid production by *Rhodotorula glutinis* cultivated on waste materials

In Table 23, Table 24, Table 25 and *Table 26* and *Graph 11*, *Graph 12*, *Graph 13* and *Graph 14*, results of *R. glutinis* biomass and carotenoid production on liquid or processed whey, potato extract, apple mass and various cereal waste substrates (for sample descriptions please see chapter 6.2.1.) are summarized.

- production of β-carotene by *R. glutinis* cultivated on whey and potato extract

Table 23: Carotenoid production by *R. glutinis* cultivated on whey and potato extract

	Cell dry weight (g/l)	β - carotene (mg/l culture fluid)	β - carotene (μg/g CDW)
wt 1	8.96	6.22	693.8
WL 1	5.84	12.48	2137.1
WLA 1	9.05	22.84	2524.8
Wl 1	4.47	0.60	135.0
PE 1	7.26	15.22	2096.2
wt 2	7.03	4.51	640.6
WL 2	8.13	17.35	2133.1
WLA 2	8.12	33.16	4082.8
Wl 2	4.52	0.09	20.2
wt 3	7.66	6.82	889.7
PE 3	5.51	12.08	2191.4



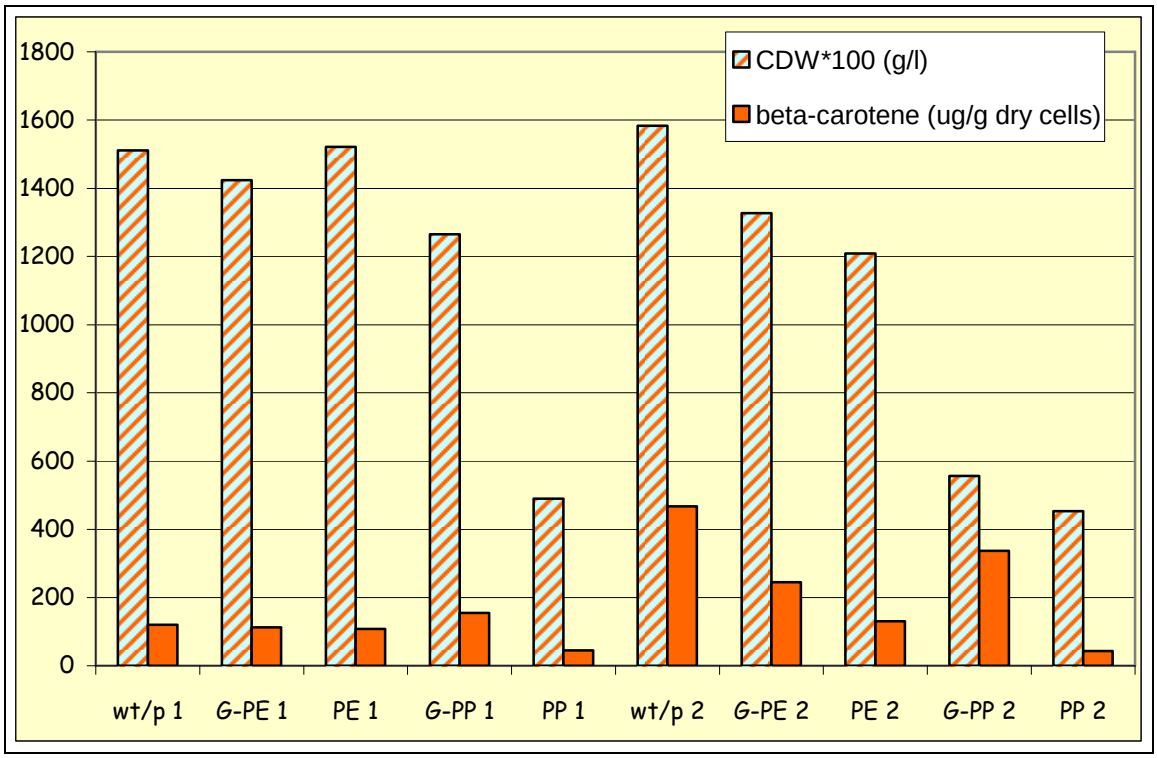
Graph 11: Carotenoid production by *R. glutinis* cultivated on whey and potato extract

Cellular accumulation of β-carotene in yeast *Rhodotorula glutinis* was the highest among all tested strains. In *Rhodotorula glutinis* addition of non-processed lyophilized whey into production medium led to about 3-times increase in β-carotene production. β-carotene formation in media with deproteinated whey was even higher. Deproteinated lyophilized whey added into both, inoculation and production media, appeared to be the most suitable for carotenogenesis in *Rhodotorula glutinis* (12.48 mg of β-carotene/l culture fluid; 2137.1 μg of β-carotene/g CDW) (See *Table 23* and *Graph 11*).

- β-carotene production by *R. glutinis* cultivated on potato mass

Table 24: Carotenoid production by *R. glutinis* cultivated on potato mass

	Cell dry weight (g/l)	β - carotene (mg/l culture fluids)	β - carotene (μg/g CDW)
wt/p 1	15.12	1.81	119.5
G-PE 1	14.24	1.60	112.6
PE 1	15.22	1.64	107.8
G-PP 1	12.65	1.96	154.6
PP 1	4.90	0.22	44.9
wt/p 2	15.84	7.41	467.9
G-PE 2	13.27	3.24	244.1
PE 2	12.09	1.58	130.7
G-PP 2	5.56	1.88	337.6
PP 2	4.54	0.19	42.9



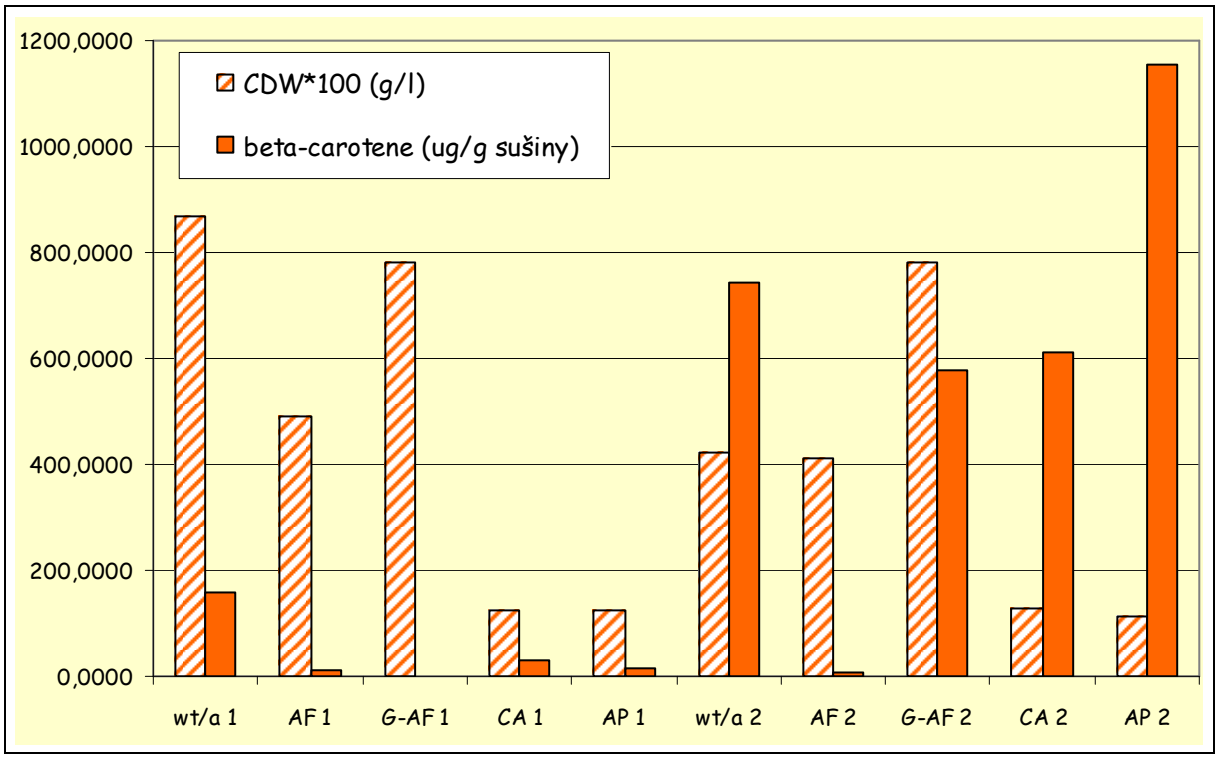
Graph 12: Carotenoid production by *R. glutinis* cultivated on potato mass

The β-carotene production by *Rhodotorula glutinis* on potato mass appeared comparable with that on glucose medium. In medium with potato extract added into inoculation medium, it is even 4-times higher (See *Table 24* and *Graph 12*). Low cost and the easy availability of this carbohydrate source make from it promising and worthwhile substrate for carotenoid production.

- β-carotene production by *R. glutinis* cultivated on apple mass

Table 25: Carotenoid production by *R. glutinis* cultivated on apple mass

	Cell dry weight (g/l)	β - carotene (mg/l culture fluids)	β - carotene (μg/g CDW)
wt/a 1	8.70	1.36	156.6
AF 1	4.90	0.06	11.5
G-AF 1	7.82	0.01	1.6
AC 1	1.25	0.04	31.2
AP 1	1.25	0.02	15.7
wt/a 2	4.23	3.14	742.4
AF 2	4.13	0.04	8.9
G-AF 2	7.82	4.51	575.9
AC 2	1.30	0.79	610.5
AP 2	1.15	1.32	1154.2



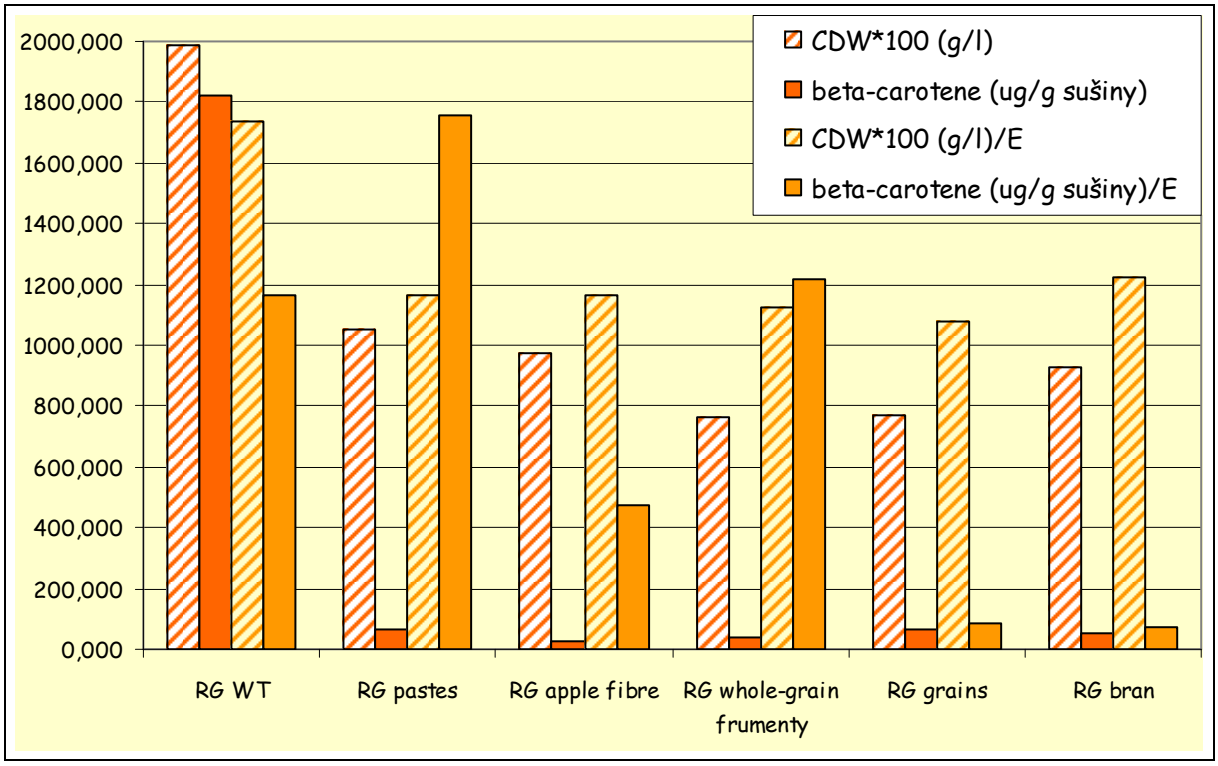
Graph 13: Carotenoid production by R. glutinis cultivated on apple mass

Carotenogenesis by *Rhodotorula glutinis* cultivated on apple mass appeared to be significant only when apple mass is added also into inoculation media. It seems that the yeast needs firstly adapt to this nutrient source. The best results were achieved when apple fiber was used as carbon source in conjunction with glucose (See *Table 25* and *Graph 13*).

- β-carotene production by *Rhodotorula glutinis* cultivated on various cereal based materials

Table 26: Carotenoid production by *R. glutinis* cultivated on various cereal-based substrates

		Cell dry weight (g/l)	β - carotene (mg/l culture fluid)	β - carotene (μg/g CDW)
wt	-	19.84	36.14	1821.1
	E	17.38	20.20	1162.3
Pastes	-	10.50	0.72	68.7
	E	11.68	20.53	1757.9
AF	-	9.72	0.28	28.9
	E	11.68	5.56	476.1
WGF	-	7.62	0.31	40.4
	E	11.27	13.71	1216.7
Grains	-	7.72	0.49	62.9
	E	10.80	0.91	84.1
Bran	-	9.26	0.48	51.6
	E	12.24	0.85	69.3



Graph 14: Carotenoid production by *R. glutinis* cultivated on various cereal-based substrates

Cereal materials contain complex carbohydrates which are utilizable only by microorganism employed by appropriate hydrolytic enzymes. Their exploitation for carotenogenesis by red yeast is able only if they are pretreated by these types of enzymes. In this work, preliminary set of experiments using extracellular extract of mould *Fusarium solani* to digestion of mentioned materials was performed. The β-carotene production by *Rhodotorula glutinis* on predigest cereal materials was then examined. It seems that pastes and whole-grain frumenty could be the promising substrates for carotenogenesis. However the β-carotene determination could be incorrect because of dark red colour of mould culture. Therefore, the assessment of these materials for carotenoid production needs next optimization.

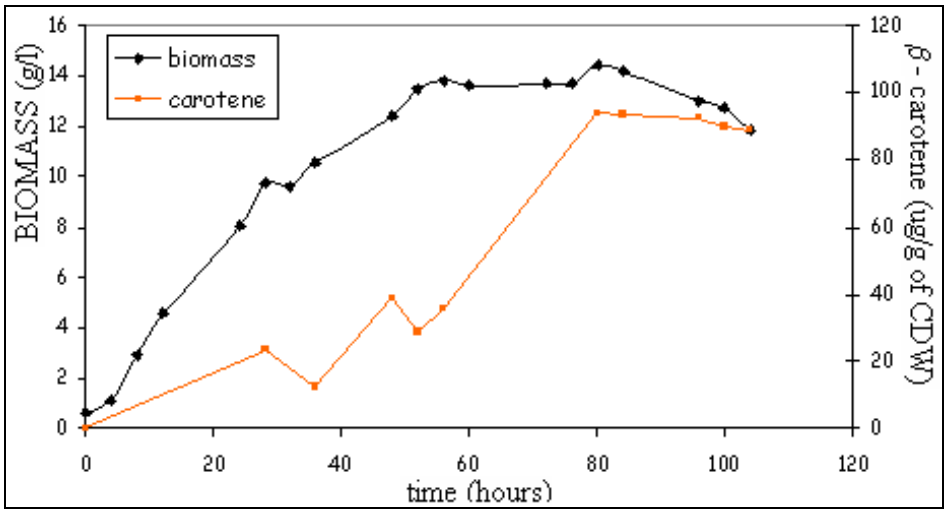
6.3.5. Carotenoid production by red yeast *Rhodotorula rubra*

6.3.5.1. Carotenoid production by *Rhodotorula rubra* cultivated in optimal conditions

In *Table 27* are summarized results of *R.rubra* growth data and β-carotene production. Similarities of biomass and β-carotene production are illustrated in *Graph 15*.

Table 27: Biomass and carotenoid production by *Rhodotorula rubra*

cultivation time (hours)	biomass (g/l)	β-carotene (µg/g CDW)
0	0	0
4	1.11	-
8	2.91	-
12	4.54	-
24	8.04	-
28	9.77	23.24
32	9.59	-
36	10.55	12.12
48	12.38	38.68
52	13.46	28.41
56	13.81	35.93
60	13.64	-
72	13.69	-
76	13.69	-
80	14.45	94.01
84	14.15	93.49
96	12.99	92.29
100	12.70	89.70
104	11.84	88.80



Graph 15: Carotenoid production by *Rhodotorula rubra*

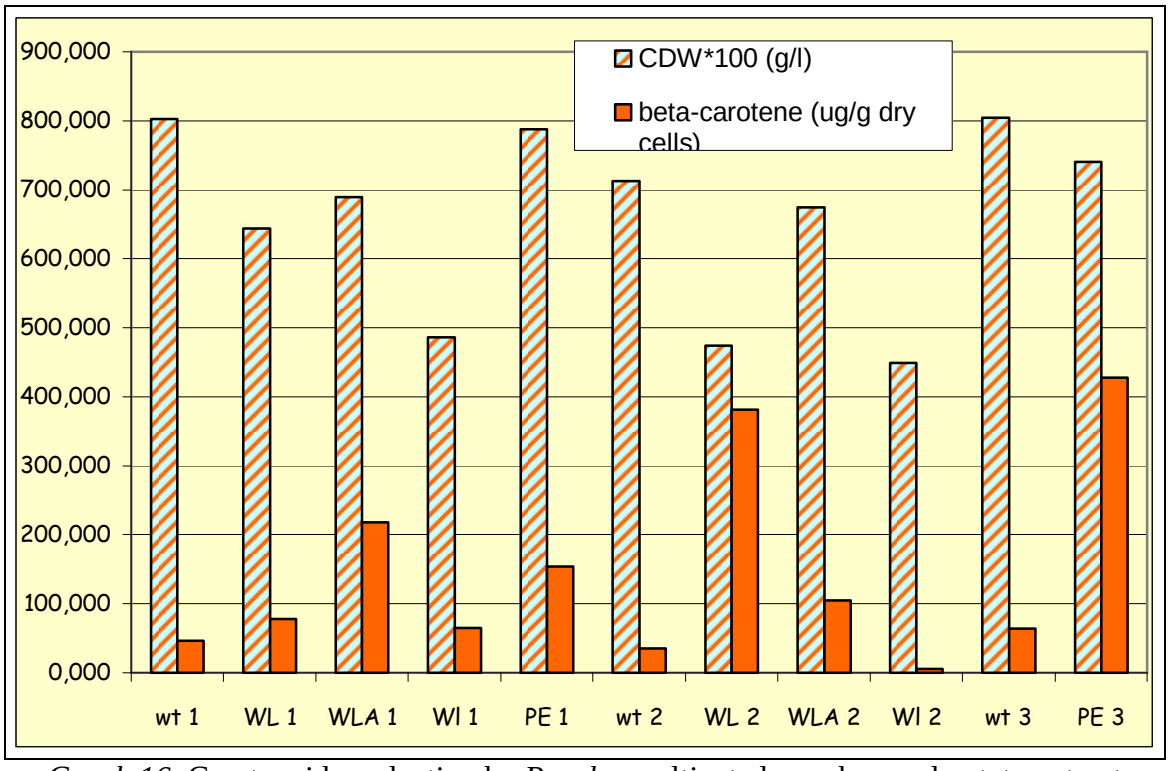
The highest carotenoid production in yeast *Rhodotorula rubra* cultivated under optimal conditions was obtained in 80th hour of cultivation (94.01 µg/g cell dry mass).

6.3.5.2. Carotenoid production by *Rhodotorula rubra* cultivated on whey and potato extract

In *Table 28* and *Graph 16*, results of *R. rubra* biomass and pigment production on liquid or processed whey and potato extract (for sample descriptions please see chapter 6.2.1.) are summarized.

Table 28: Carotenoid production by *R. rubra* cultivated on whey and potato extract

	Cell dry weight (g/l)	β - carotene (mg/l culture fluid)	β - carotene (μg/g CDW)
wt 1	8.02	0.37	46.3
WL 1	6.44	0.50	77.7
WLA 1	6.89	1.50	218.0
Wl 1	4.87	0.31	64.5
PE 1	7.88	1.21	153.8
wt 2	7.13	0.25	35.0
WL 2	4.74	1.81	381.7
WLA 2	6.74	0.71	104.9
Wl 2	4.49	0.03	5.9
wt 3	8.04	0.52	64.4
PE 3	7.41	3.17	427.9



Graph 16: Carotenoid production by *R. rubra* cultivated on whey and potato extract

Maximal β-carotene yields in the yeast *Rhodotorula rubra* were obtained in media with non-processed lyophilized whey added into inoculation and production media (8-times higher β-carotene formation than in wildtype; 1.81 mg of β-carotene/l culture fluid; 381.7 μg of β-carotene/g CDW) and in media with potato extract also added into both media (9-times higer than in wildtype; 3.17 mg of β-carotene/l culture fluid; 427.9 μg of β-carotene/g CDW) (See *Table 28* and *Graph 16*).

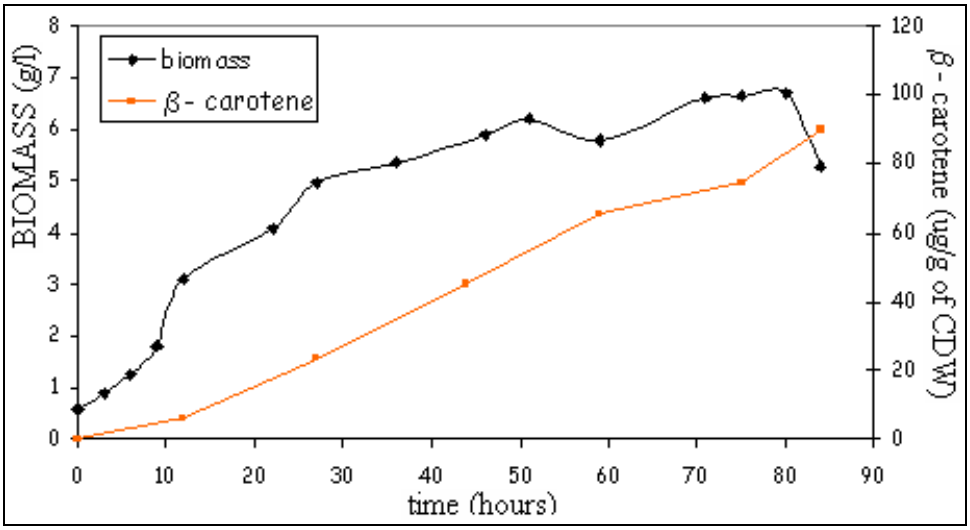
6.3.6. Carotenoid production by red yeast *Sporobolomyces roseus*

6.3.6.1. Carotenoid production by *Sporobolomyces roseus* cultivated in optimal conditions

In *Table 29*, results of *S.roseus* growth data and β-carotene production are summarized. Similarities of growth curve and carotene-production course are illustrated in *Graph 18*.

Table 29: Biomass and carotenoid production by *Sporobolomyces roseus*

cultivation time (hours)	biomass (g/l)	β-carotene (μg/g CDW)
0	0.58	0
3	0.88	-
6	1.25	-
9	1.80	-
12	3.11	5.89
22	4.08	-
27	4.96	23.68
36	5.36	-
44	-	44.86
46	5.89	-
51	6.19	-
59	5.80	65.46
71	6.60	-
75	6.64	74.72
80	6.71	-
84	5.26	90.09



Graph 17: Carotenoid production by *Sporobolomyces roseus*

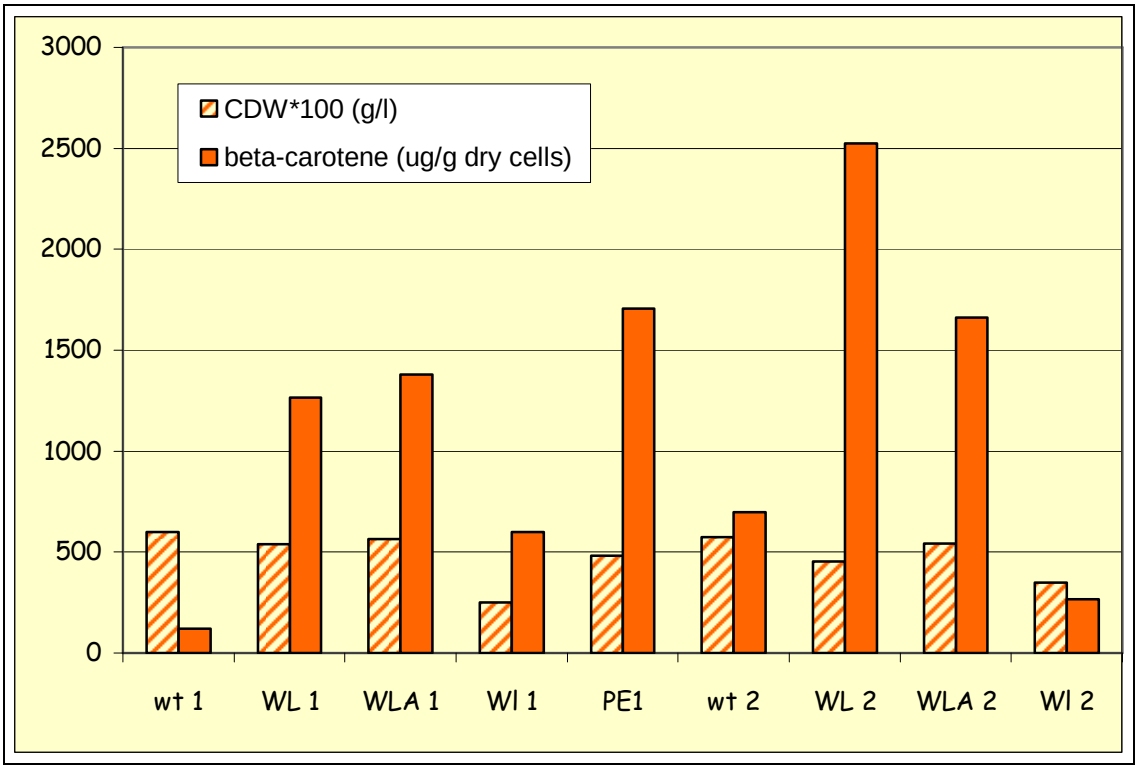
Maximal yield of carotenoid in yeast Sporobolomyces roseus was reached in 84th hour of cultivation (90.09 μg/g cell dry mass).

6.3.6.2. Carotenoid production by *Sporobolomyces roseus* cultivated on whey

In *Table 30* and *Graph 18*, results of *S.roseus* biomass and carotenoid production on liquid or processed whey and potato extract (for sample descriptions please see chapter 6.2.1.) are summarized.

Table 30: Carotenoid production by *S. roseus* cultivated on whey and potato extract

	Cell dry weight (g/l)	β - carotene (mg/l culture fluid)	β - carotene (μg/g dry cells)
wt 1	5.98	0.72	120.5
WL 1	5.39	6.83	1265.9
WLA 1	5.65	7.80	1380.2
Wl 1	2.51	1.50	598.8
PE 1	4.82	8.22	1705.9
wt 2	5.75	4.01	696.4
WL 2	4.55	11.47	2522.8
WLA 2	5.43	9.02	1660.7
Wl 2	3.49	0.92	264.8



Graph 18: Carotenoid production by *S. roseus* cultivated on whey and potato extract

20-fold increase of β-carotene production when compared with wildtype was observed in yeast *Sporobolomyces roseus* cultivated on non-processed lyophilized whey added into inoculation and production media (11.47 mg of β-carotene/l culture fluid; 2522.8 μg of β-carotene/g CDW). High cellular accumulation of β-carotene was achieved also on deproteinated lyophilized whey added either only in production media (1380.2 μg of β-carotene/g CDW) or in both, inoculation and production media (1660.7 μg of β-carotene/g CDW), and on potato extract (1705.9 μg of β-carotene/g CDW) (See *Table 30* and *Graph 18*).

6.4. Ergosterol production by red yeast cultivated on various waste materials

Carotenogenic yeasts are reported to accumulate also considerable amount of other lipidic compounds, e.g. sterols. Production of sterols can be strongly influened by cultivation conditions including stress (See chapter 3.3.5.6.). In this work, ergosterol production by red yeast cultivated on various waste materials was studied. Ergosterol production was quantified against peak area calibrations obtained from standard curve:

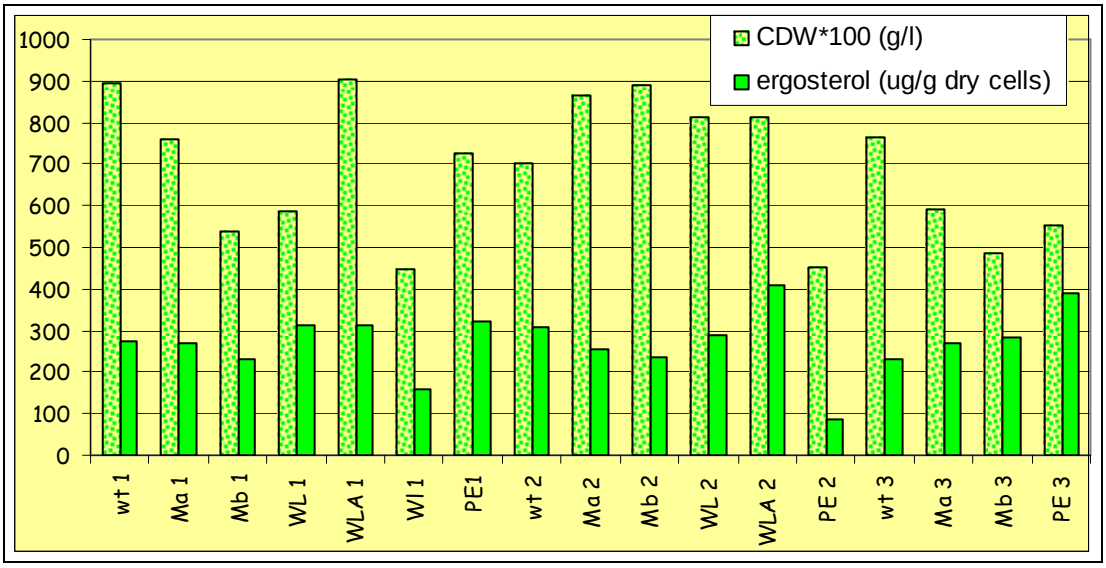
$$A[mV.s] = 64.4 \times c_{ergosterol}\ [\mu g/ml]$$

6.4.1. Ergosterol production by red yeast *Rhodotorula glutinis*

In *Table 31* and *Graph 19*, results of ergosterol production in *R.glutinis* cells cultivated on liquid or processed whey and potato extract (for sample descriptions please see chapter 6.2.1.) are presented.

Table 31: Ergosterol production by *R. glutinis* cultivated on whey and potato extract

	Cell dry weight (g/l)	ergosterol (mg/l culture fluid)	ergosterol (µg/g CDW)
wt 1	8.965	24.51	273.36
Ma 1	7.578	20.32	268.10
Mb 1	5.400	12.53	231.97
WL 1	5.842	18.34	313.99
WLA 1	9.047	28.48	314.83
Wl 1	4.465	6.98	156.39
PE 1	7.259	23.22	319.91
wt 2	7.033	21.65	307.85
Ma 2	8.646	22.24	257.21
Mb 2	8.882	21.13	237.90
WL 2	8.133	23.30	286.47
WLA 2	8.122	33.06	407.04
Wl 2	4.517	3.94	87.16
wt 3	7.660	17.82	232.67
Ma 3	5.914	16.05	271.43
Mb 3	4.856	13.73	282.82
PE 3	5.513	21.56	391.06



Graph 19: Ergosterol production by *R. glutinis* cultivated on whey and potato mass

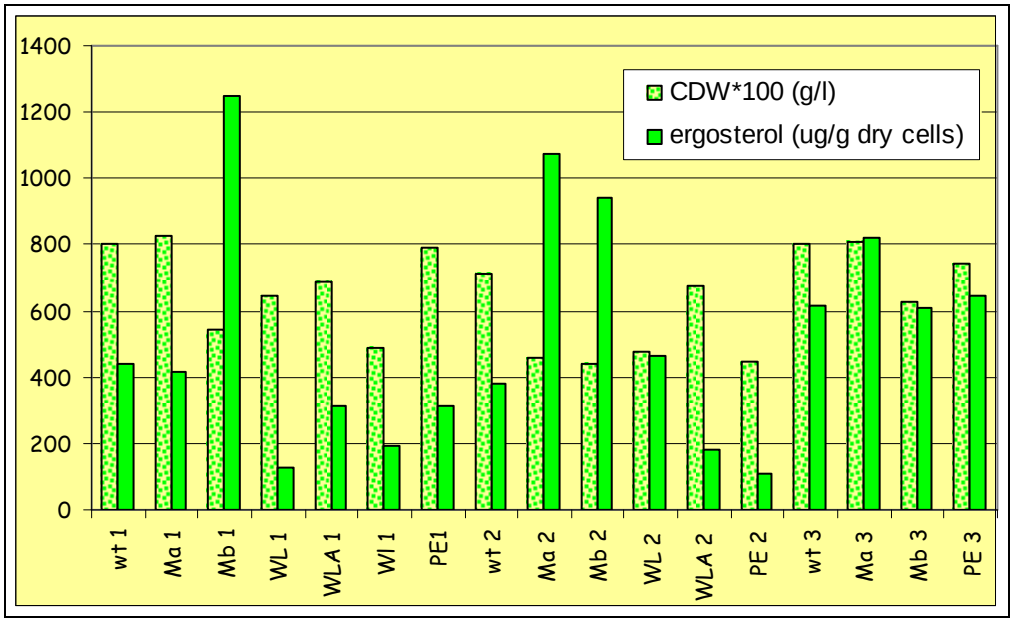
In *Rhodotorula glutinis* cells, lyophillized deproteinated whey added only into production medium or into both, inoculation and production media, was chosen as the most appropriate way for ergosterol production.

6.4.2. Ergosterol production by red yeast *Rhodotorula rubra*

In *Table 32* and *Graph 20*, results of ergosterol production in *R. rubra* cells cultivated on liquid or processed whey and potato extract (for sample descriptions please see chapter 6.2.1.) are summarized.

Table 32: Ergosterol production by *R. rubra* cultivated on whey and potato extract

	Cell dry weight (g/l)	ergosterol (mg/l culture fluid)	ergosterol (µg/g CDW)
wt 1	8.023	35.39	441.13
Ma 1	8.293	34.31	413.70
Mb 1	5.448	68.03	1248.73
WL 1	6.435	8.09	125.76
WLA 1	6.892	21.68	314.52
Wl 1	4.865	9.36	192.45
PE 1	7.875	24.74	314.19
wt 2	7.127	27.30	383.06
Ma 2	4.569	48.97	1071.77
Mb 2	4.387	41.27	940.90
WL 2	4.739	22.00	464.25
WLA 2	6.744	12.29	182.25
Wl 2	4.491	4.88	108.64
wt 3	8.040	49.32	613.34
Ma 3	8.084	66.53	822.93
Mb 3	6.301	38.24	606.96
PE 3	7.405	47.77	645.13



Graph 20: Ergosterol production by *R. rubra* cultivated on whey and potato extract

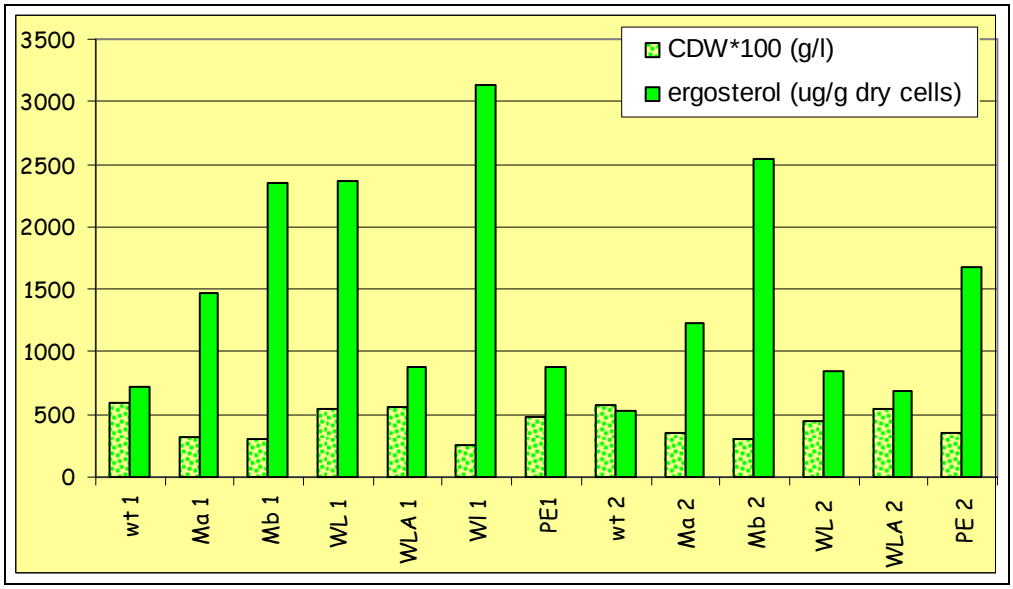
The highest ergosterol accumulation by red yeast *Rhodotorula rubra* was obtained for cultivation in production medium b and for cultivation in inoculation and production medium a and b. Maximal yields were also in media a and b. These media were shown to be appropriate for biomass and ergosterol production in carotenogenic yeast.

6.4.3. Ergosterol production by red yeast *Sporobolomyces roseus*

In *Table 33* and *Graph 21*, results of ergosterol production in *R. rubra* cells cultivated on liquid or processed whey (for sample descriptions please see chapter 6.2.1.) are summarized.

Table 33: Ergosterol production by *S. roseus* cultivated on whey

	Cell dry weight (g/l)	ergosterol (mg/l culture fluid)	ergosterol (µg/g CDW)
wt 1	5.984	4.30	0.72
Ma 1	3.143	4.60	1.46
Mb 1	3.085	7.24	2.35
WL 1	5.394	12.76	2.37
WLA 1	5.654	4.95	0.88
Wl 1	2.506	7.85	3.13
PE 1	4.819	4.26	0.88
wt 2	5.753	3.04	0.53
Ma 2	3.440	4.25	1.24
Mb 2	3.052	7.74	2.54
WL 2	4.547	3.84	0.84
WLA 2	5.431	3.69	0.68
Wl 2	3.489	5.84	1.67



Graph 21: Ergosterol production by *S. roseus* cultivated on whey

In yeast *Sporobolomyces roseus*, the highest ergosterol accumulation was observed in yeast cultivated in liquid whey (3.13 µg/g of CDW). However the yeast growth in this media was very low. The best result of ergosterol production was achieved in media with lyophillized, non-processed whey (12.76 mg/l culture fluid).

Carotenoid yields obtained in this work appeared comparable with those reported in the literature (See *Table 34*). Also ergosterol production by carotenogenic yeasts is significant (See chapter 6.4.). Mentioned compounds increase biological value and so exploitation of the yeast. This carotene-enriched biomass can be used as source of carotenoid pigments or as animal feed with biologically valuable compounds.

Table 34: Comparison of carotenoid production by *Rhodotorula species* cultivated on different waste substrates

Rhodotorula species	Carbon source	Cultivation process	Cell mass (g/l)	Carotenoids (mg/g dry cells)	Carotenoids (mg/l culture)	References
R. glutinis	WLA 2	batch	8.12	8.20	66.32	this study
R. glutinis	pastes + enzymes	batch	11.68	3.60	40.10	this study
R. glutinis ATCC 26085	glucose	batch		0.206		[81]
R. glutinis 32	glucose	batch	23.90	5.40	129.00	[79]
R. glutinis 32	sugar cane molasses	fed-batch	78.00	2.36	183.00	[79]
R. glutinis DBVPG 3853 *D. castellii* DBVPG 3503	corn syrup	fed-batch	15.30	0.54	8.20	[80]
R. glutinis TISTR	hydrolyzed mung bean waste flour	batch	10.35	0.35	3.48	[78]
R. glutinis 22P *L. helveticus* 12A	whey ultrafiltrate	batch	30.20	0.27	8.10	[61]
R. mucilaginosa NRRL-2502	sugar-beet molasses	batch	4.20	21.20	89.0	[76]
R. mucilaginosa NRRR-2502	whey	batch	2.40	29.20	70.0	[76]

In the future, other types of waste materials (for instance from winemarket) are intended to be tested as carbon sources for carotenogenesis in red yeasts. Moreover application of an environmental stress in combination with waste materials can lead to overproduction of carotenoids and lipids and decrease cost of their production. Such strategies could result into production of yeast biomass rich not only in carotenoids and other provitamins, but also in other nutrition components (proteins, PUFA, metal inos etc.) that originate both from yeast cells and from cultivation substrates. This is the way to production of complex food additives based on naturally enriched yeast biomass.

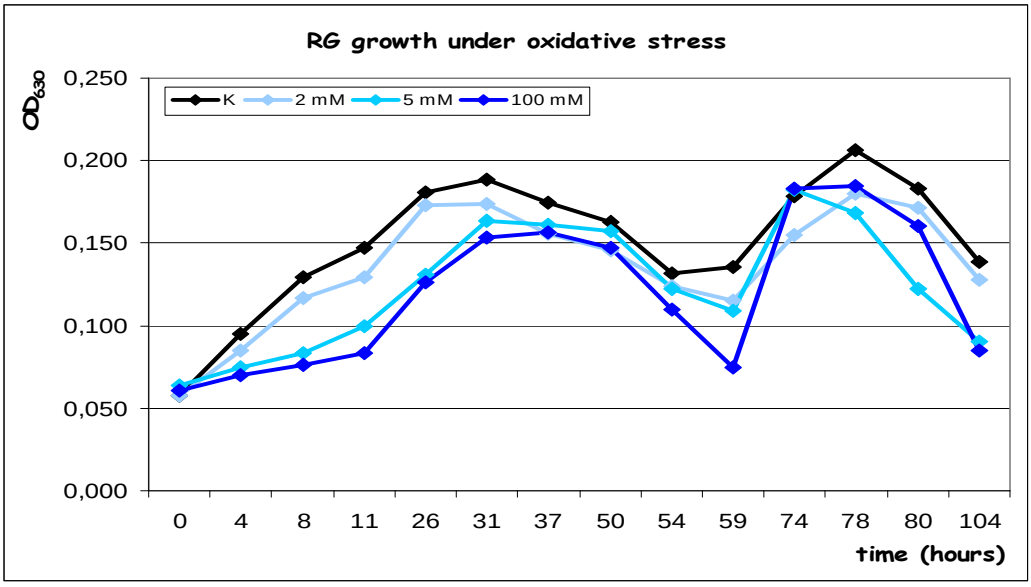
6.5. Effect of environmental stress on yeast growth

Oxidative and osmotic stress was reported to induce carotenoid production as part of red yeast stress response. To enable a functional genomics modeling of gene–environment interactions, quantitative assays are required both for gene expression and for the phenotypic responses to environmental challenge. Phenotypic profiling of the oxidative and osmotic stress responses demonstrates genetic susceptibility of yeast to environmental stress. Measurements of cell viability and growth provide versatile and sensitive assays for characterization of cytotoxic effect of environmental stress. Yeast cells exhibit a graded concentration-dependent response to chemically induced stress: continued growth, cellular adaptation, checkpoint arrest/growth delay, apoptosis, and necrosis. Standardized assay conditions are required to quantify yeast growth curves (See chapter 3.3.5.1.).

6.5.1. Effect of oxidative stress on yeast growth

Table 35: Growth responses of *Rhodotorula glutinis* exposed to hydrogen peroxide

time of cultivation (hours)	0	4	8	11	26	31	37	50	54	59	74	78	80	104
WT	0.066	0.098	0.133	0.149	0.177	0.187	0.165	0.158	0.132	0.135	0.167	0.201	0.183	0.145
2 mM H_2O_2	0.060	0.085	0.120	0.129	0.175	0.174	0.153	0.148	0.130	0.120	0.168	0.185	0.171	0.144
5 mM H_2O_2	0.067	0.079	0.084	0.100	0.139	0.153	0.152	0.151	0.122	0.102	0.168	0.182	0.124	0.098
100 mM H_2O_2	0.066	0.078	0.083	0.091	0.138	0.151	0.152	0.149	0.117	0.086	0.168	0.185	0.159	0.096



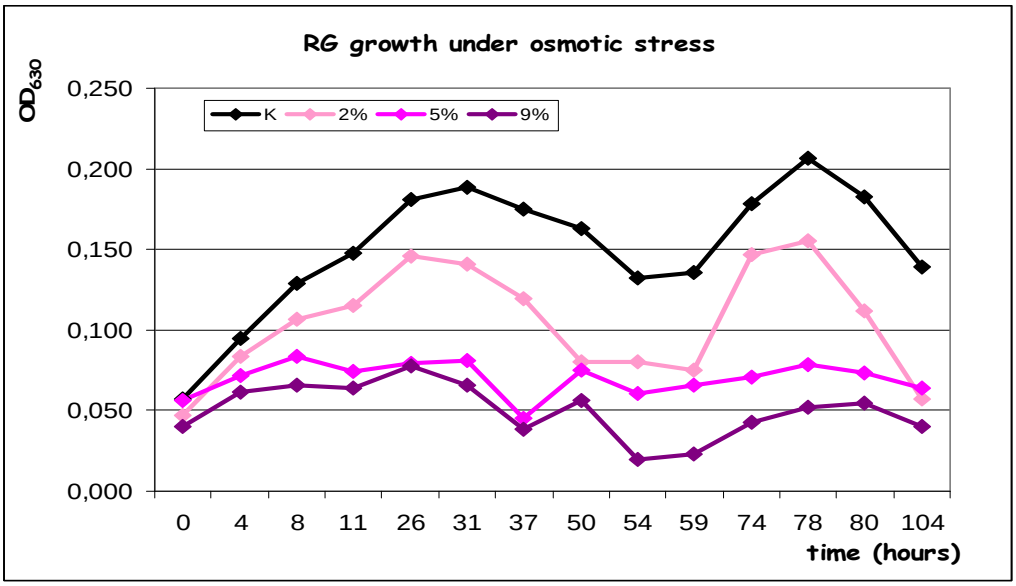
Graph 22: Growth responses of yeast *Rhodotorula glutinis* exposed to hydrogen peroxide

In this experiment, the growth delay response increases with the concentration of hydrogen peroxide over the range of 2 – 100 mM exposure.

6.5.2. Effect of osmotic stress on yeast growth

Table 36: Growth responses of *Rhodotorula glutinis* exposed to osmotic stress

time of cultivation (hours)	0	4	8	11	26	31	37	50	54	59	74	78	80	104
WT	0.056	0.098	0.133	0.149	0.177	0.187	0.165	0.158	0.132	0.135	0.167	0.201	0.183	0.145
2% NaCl	0.050	0.085	0.107	0.112	0.149	0.143	0.121	0.083	0.083	0.082	0.150	0.153	0.109	0.058
5% NaCl	0.058	0.073	0.084	0.080	0.081	0.081	0.049	0.081	0.079	0.080	0.080	0.081	0.080	0.080
10% NaCl	0.044	0.058	0.058	0.058	0.081	0.059	0.045	0.053	0.023	0.023	0.043	0.050	0.051	0.041



Graph 23: Effect of osmotic stress on *R. glutinis* growth

Yeast *Rhodotorula glutinis* cultivated under osmotic stress induced by 2 % NaCl exhibits a growth delay versus the unexposed control cultures. In contrast, at exposure concentrations equal to or greater than 5 % of NaCl, the challenged cells respond by growth arrest and apoptosis.

According to our results, low concentrations of oxidative and osmotic stress, which can under specific conditions induce carotenogenesis, have no significant effect on yeast growth. However, the mentioned results are just pivot because the adaptation and growth delay responses are diffcult to measure reproducibly in shake flask cultures. This problem can be alleviated by an automated cell growth assay in a microculture reported by Weiss at all [83]. Yeast cells in the microculture format maintain uniform growth conditions that enable highly precise and reproducible assays of chemical sensitivity.

6.6. Effect of environmental stress on yeast morphology

Morphological changes are visible indicators of microbial adaptability to environmental variations. Microscopic observation of morphology of yeast exposed to environmental stress can provide futher information on cytotoxicity of environmental stress.

6.6.1. Morphology of yeast cultivated under oxidative and osmotic stress

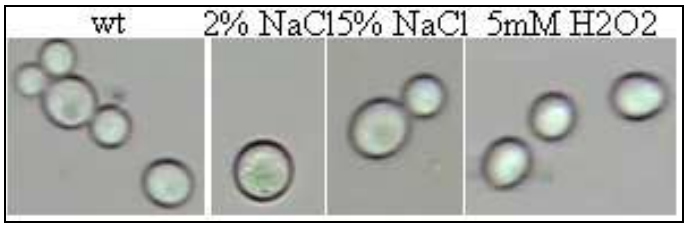


Figure 48: Morphological changes in *R. glutinis* exposed to osmotic and oxidative stress

Two predominant morphotypes appear in *Rhodotorula glutinis* exposed to environmental stress: normal ellipsoidal and spherical forms (See chapter 3.4.1.1.). Ellipsoidal shaped cells are the majority in control and under oxidative stress. Spherical cells are obtained in the presence of osmotic stress. The presence of dividing cells confirms the persistence of normally metabolizing yeast. Thicker cell wall is observed in cells exposed to osmotic or oxidative stress. This can be caused by higher content of carotenoid produced by stressed yeasts. Similar results are obtained also for other carotenogenic yeasts (See next).

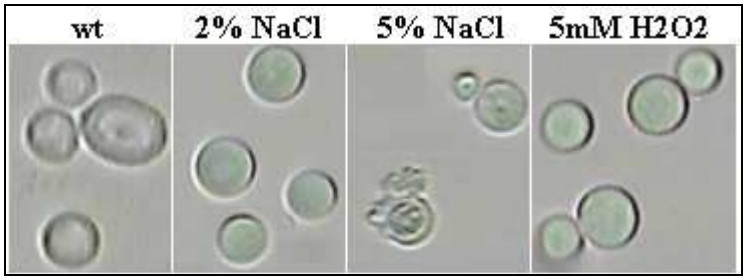


Figure 49: Morphological changes in *R. rubra* exposed to osmotic and oxidative stress

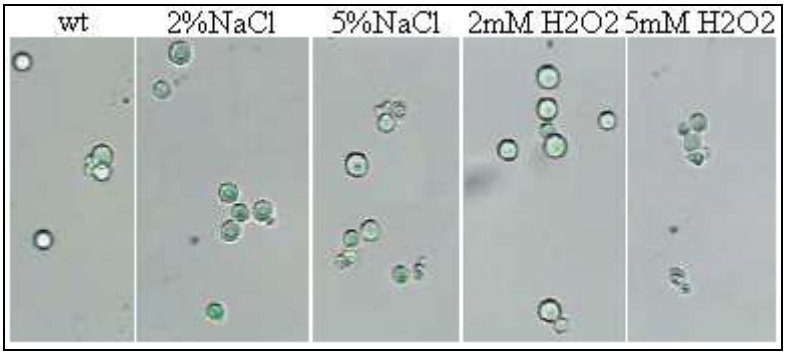


Figure 50: Morphological changes in *R. aurantiaca* exposed to osmotic and oxidative stress

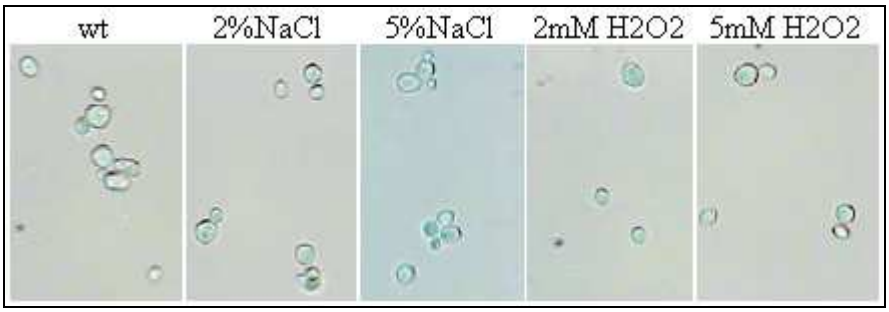


Figure 51: Morphological changes in *S. roseus* exposed to osmotic and oxidative stress

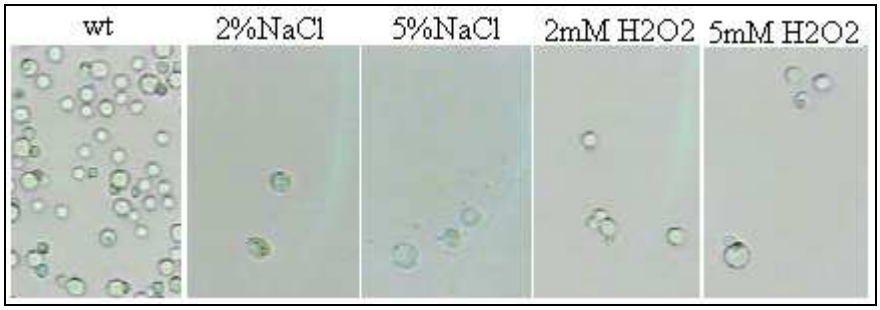


Figure 52: Morphological changes in *S. salmonicolor* exposed to osmotic and oxidative stress

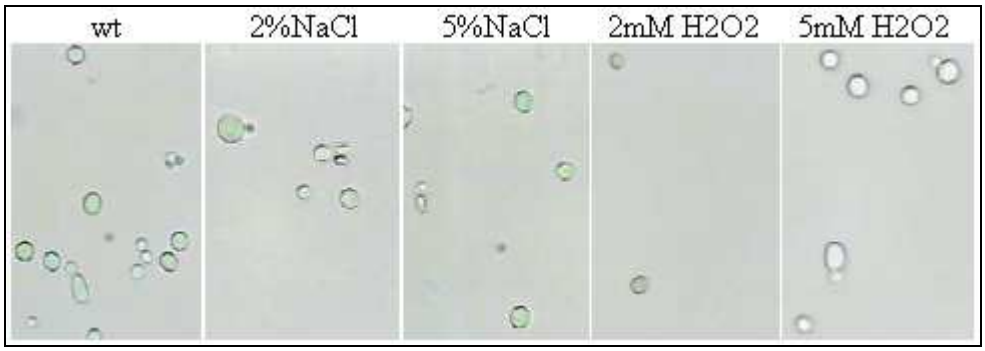


Figure 53: Morphological changes in *S. shibatanus* exposed to osmotic and oxidative stress

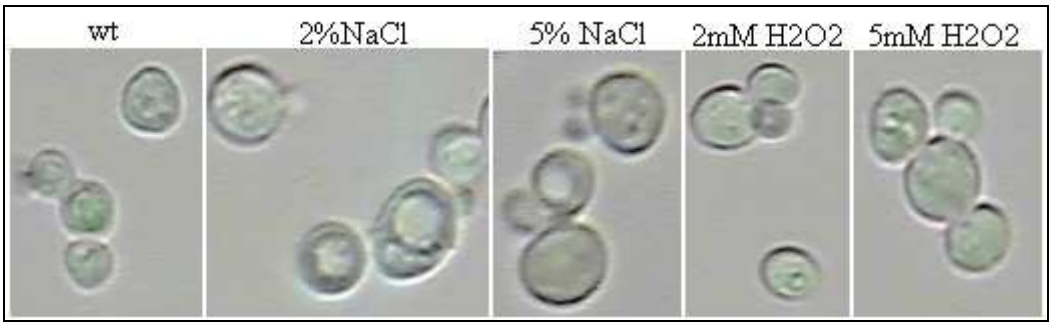


Figure 54: Morphological changes in *C. capitatum* exposed to osmotic and oxidative stress

6.6.2. Morphology of yeast cultivated in media with restricted carbon or nitrogen source

- cultivation on whey and potato mass

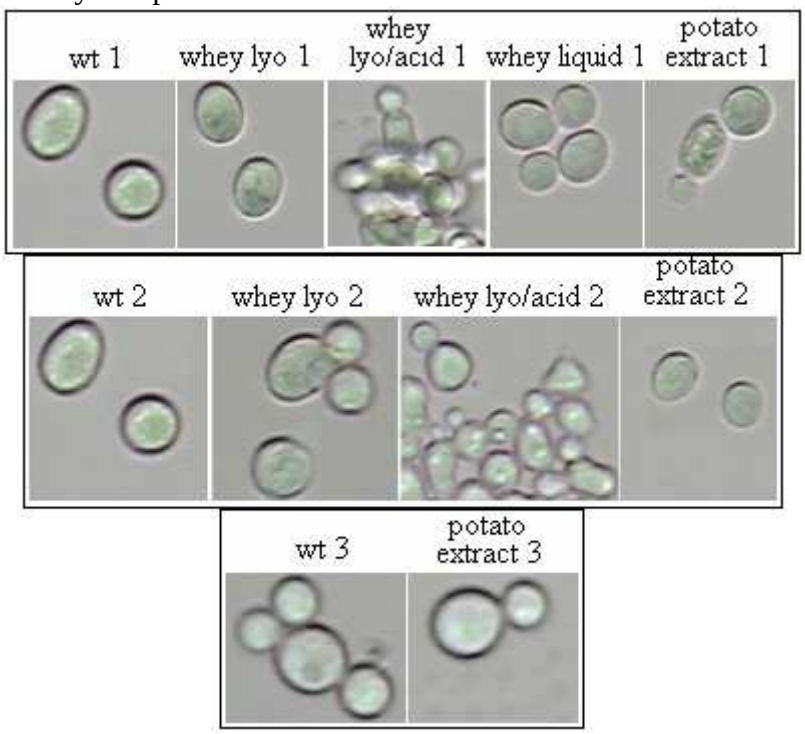


Figure 55: Morphology of *Rhodotorula glutinis* cultivated on whey and potato mass (x)

Yeast *Rhodotorula glutinis* cultivated on various forms of whey shows no significant differences in cell morphology when compared with yeast cultivated in optimal glucose medium. Agglomeration of cells was observed in media with deproteinated lyophilized whey.

- cultivation on potato mass

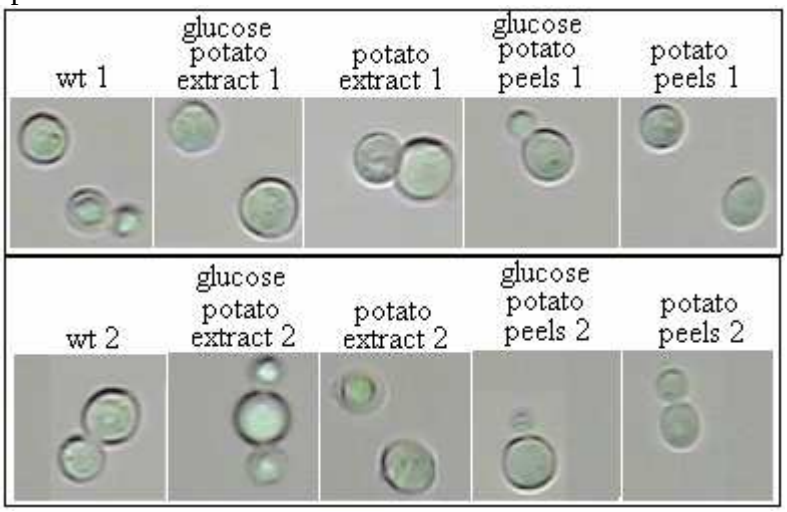


Figure 56: Morphology of *Rhodotorula glutinis* cultivated on potato mass

No morphological changes were observed in yeast *Rhodotorula glutinis* cultivated on various forms of potato mass in comparison with yeast cultivated on conventionally used glucose medium. Potato mass seems to have no deleterious effect on red yeasts.

- cultivation on apple mass

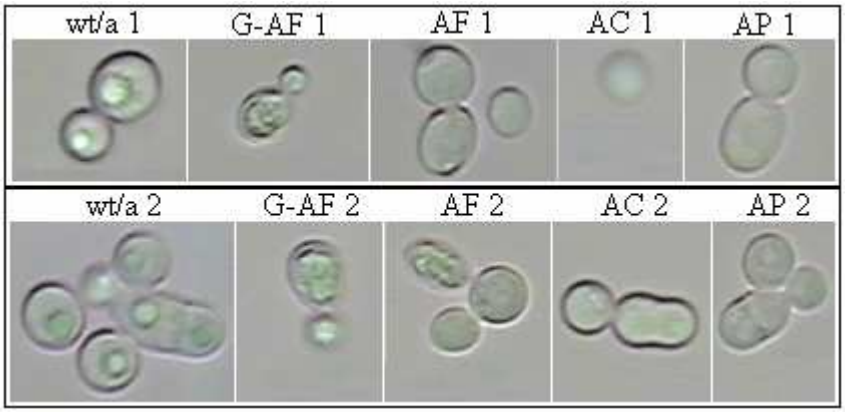


Figure 57: Morphology of *Rhodotorula glutinis* cultivated on apple mass

Elongated cells of yeast *Rhodotorula glutinis* appear in media with apple fiber or apple peels. Moreover, cells cultivated on apple mass seem to underlie dehydration; cytoplasmic membrane invaginations are observed. Apple mass likely presents a slight osmotic stress for red yeasts.

- cultivation on various cereal-based substrates

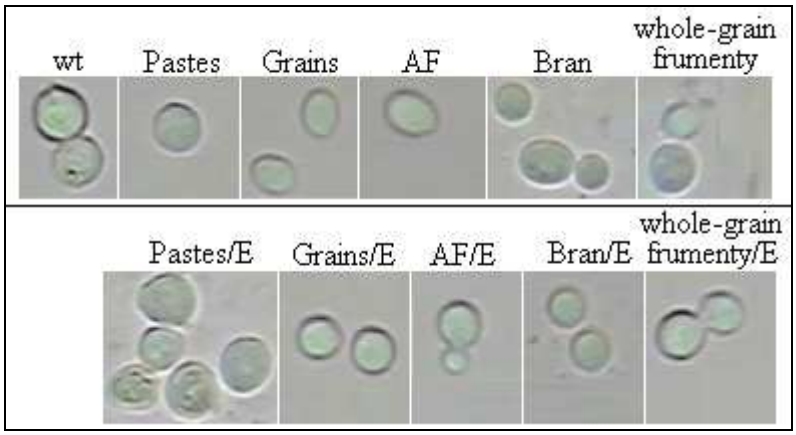


Figure 58: Morphology of *Rhodotorula glutinis* cultivated on various cereal-based substrates

Shape of yeast *Rhodotorula glutinis* cultivated on cereal-based substrates is spherical or ellipsoidal. Invaginations of cytoplasmic membrane (See chapter 3.2.2.3.) are observed in cells cultivated on pastes pretreated with hydrolytic enzymes of mould *Fusariom solani* (See *Figure 58*). This substrate makes for red yeast mild osmotic stress.

6.7. Environmental stress and ROS production

Higher ROS production in cells is an indicator of programmed cell death. An effective method of quantification of ROS production in living cells is so required. Due to the inherent instability and reactivity of most ROS and their very low steady-state levels, this is much more difficult task than determination of concentration of antioxidants and of activities of antioxidant enzymes. Intracellular oxidation of dichlorofluorescein (H_2DCF) or dihydroethidium (DHE) is widely used to measure the ROS production within cells. Intracellular H_2O_2 and $O_2^{-}\cdot$ production can be detected with H_2DCF and DHE, respectively (See chapter 3.2.2.2.).

6.7.1. Oxidative stress and ROS production

DIC DHE

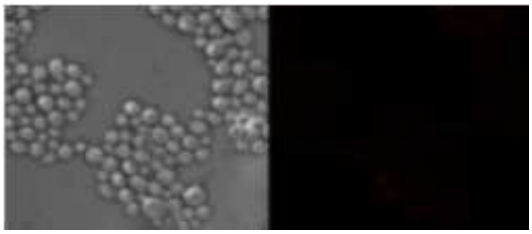


Figure 59: *R. glutinis* - wt

DIC H2DCF

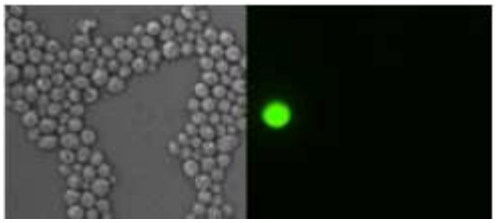


Figure 60: *R. glutinis* - wt

DIC DHE

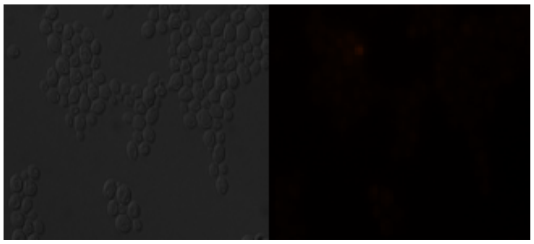


Figure 61: *R. glutinis* - 2 mM H_2O_2

DIC DHE

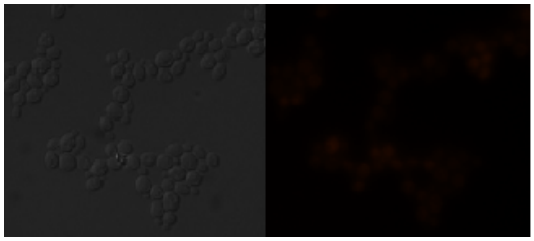


Figure 62: *R. glutinis* - 5 mM H_2O_2

DIC DHE

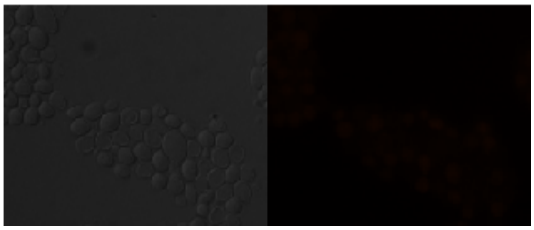


Figure 63: *R. glutinis* - 10 mM H_2O_2

DIC DHE

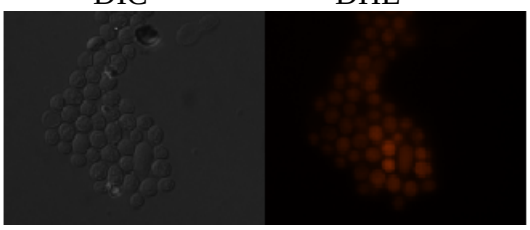


Figure 64: *R. glutinis* - 100 mM H_2O_2

DIC H2DCF

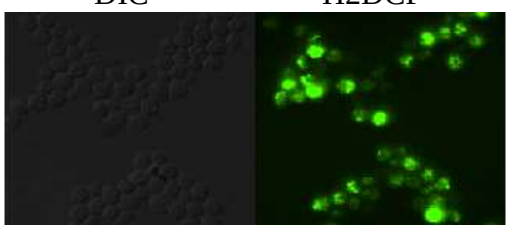


Figure 65: *R. glutinis* - 100 mM H_2O_2

Generation of $O_2^{-}\cdot$ was measured by red fluorescent cell staining due to oxidation of DHE by $O_2^{-}\cdot$. No $O_2^{-}\cdot$ was produced in either adapted or in control cells. $O_2^{-}\cdot$ formation was observed only in cells treated with 100 mM H_2O_2.

Production of H_2O_2 occurred in cells cultivated under strong oxidatve stress (100 mM H_2O_2), as shown by the presence of intense green fluorescence stained cells, due to H_2O_2-dependent oxidation of H_2DCF to DCF. In control cells no H_2O_2 generation was observed, thus suggesting that its production is specifically caused by H_2O_2 treatment.

6.7.2. Osmotic stress and ROS production

DIC DHE

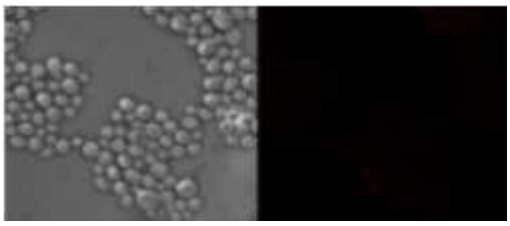


Figure 66: *R. glutinis* - wt

DIC H2DCF

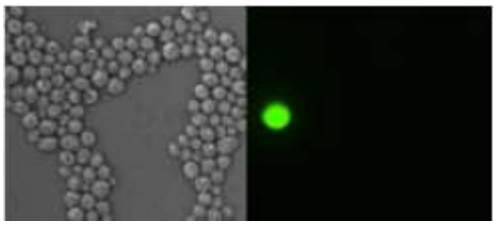


Figure 67: *R. glutinis* - wt

DIC DHE

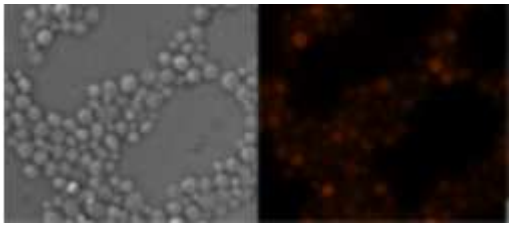


Figure 68: *R. glutinis* - 2% NaCl

DIC H2DCF

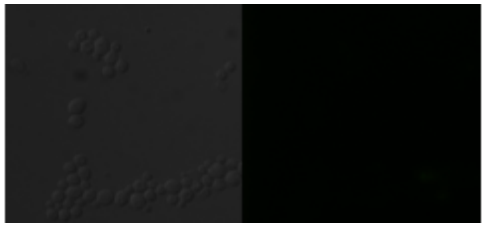


Figure 69: *R. glutinis* - 2% NaCl

DIC DHE

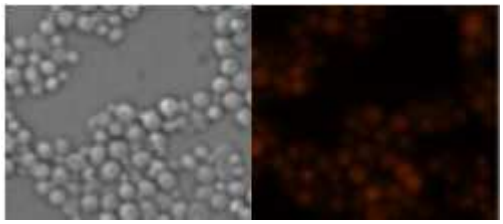


Figure 70: *R. glutinis* - 5% NaCl

DIC H2DCF

Figure 71: *R. glutinis* - 5% NaCl

DIC DHE

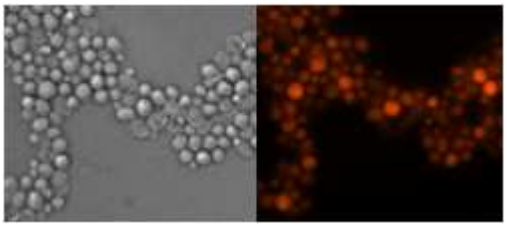


Figure 72: *R. glutinis* - 10% NaCl

DIC H2DCF

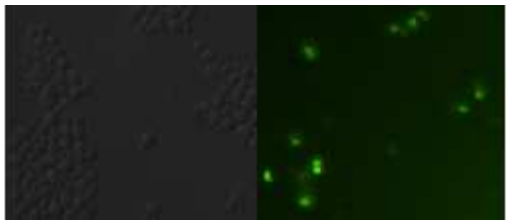


Figure 73: *R. glutinis* - 10% NaCl

Under conditions of osmotic stress, maximal $O_2^{-}\cdot$ and H_2O_2 generation was observed in cells treated with 10 % NaCl. It suggests that cells of *Rhodotorula glutinis* can adapt to lower concentrations of osmotic stress and so these concentrations can be used to stimulate carotenoid production. Under osmotic stress, ROS production can result from so called crossprotection (See chapter 3.3.1. and *Figure 14*).

6.8. Determination of DNA fragmentation

One of the most important markers of severity of environmental stress is chromosomal DNA fragmentation. Pulsed field gel electrophoresis of chromosomal DNA from cells exposed to environmetal stress cen reveal DNA breakdown into fragments of several hundred kilobases (See chapter 3.2.2.2. and 3.2.2.3.)

6.8.1. Isolation of intact chromosomal DNA – protoplast preparation

Studies on electrophoretic karotype of yeast are limited to organisms from which high quality protoplasts can be prepared in large numbers. An effective protocol for protoplast preparation is so required.

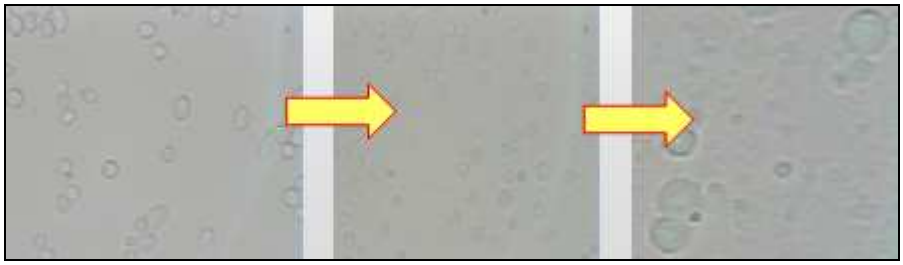


Figure 74: Protoplast preparation from model yeast *Saccharomyces cerevisiae*

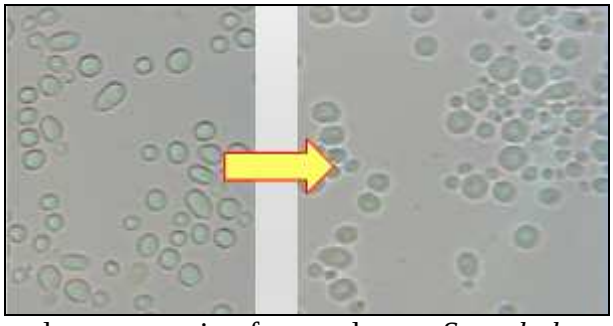


Figure 75: Protoplast preparation from red yeast *Sporobolomyces shibatanus*

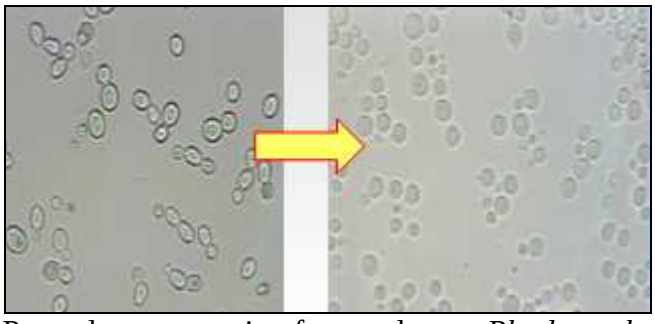


Figure 76: Protoplast preparation from red yeast *Rhodotorula aurantiaca*

Protoplasts preparation from red yeasts is very difficult due to unusual cell wall character. Different types of lytic enzymes (glycosidases and glucuronidases) and combination of several procedures is necessary to use. Protoplasts in *Rhodotorula* sp. and *Sporobolomyces* sp. were formed and can be osmotically disrupted after substantially longer period (7-9 hours) than in *Sacharomyces cerevisiae* (30 min). Isolation of protoplasts in agarose blocks led to relatively successful separation of *S. shibatanus*, *R. aurantiaca* and *S. cerevisiae* chromosomal DNA by pulsed field gel electrophoresis.

6.8.2. Pulsed Field Gel Electrophoresis optimization

- isolation of intact chromosomal DNA and karyotyping of yeast *Saccharomyces cerevisiae*

To establish a protocol for chromosome isolation and electrophoretic karyotyping of carotenogenic yeast, the method was first examined on model system *S. cerevisiae* which karyotype is well known.

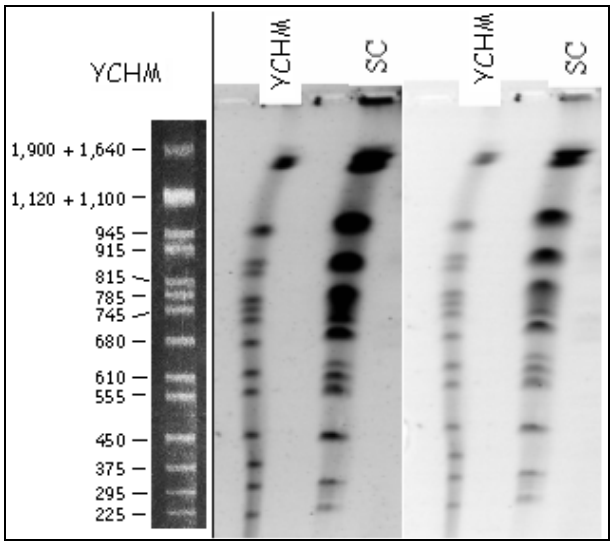


Figure 77: Pulsed Field Gel Electrophoresis of *S. cerevisiae*;
Conditions: 1.2% gel, 0.08M TBE, 162 V, 70s/15h, 100s/10h, 120s/10h, 150s/15h, 200s/15h;
Samples: YCHM – yeast chromosome marker; SC – *Saccharomyces cerevisiae*

- optimization of isolation of intact chromosomal DNA of carotenogenic yeast

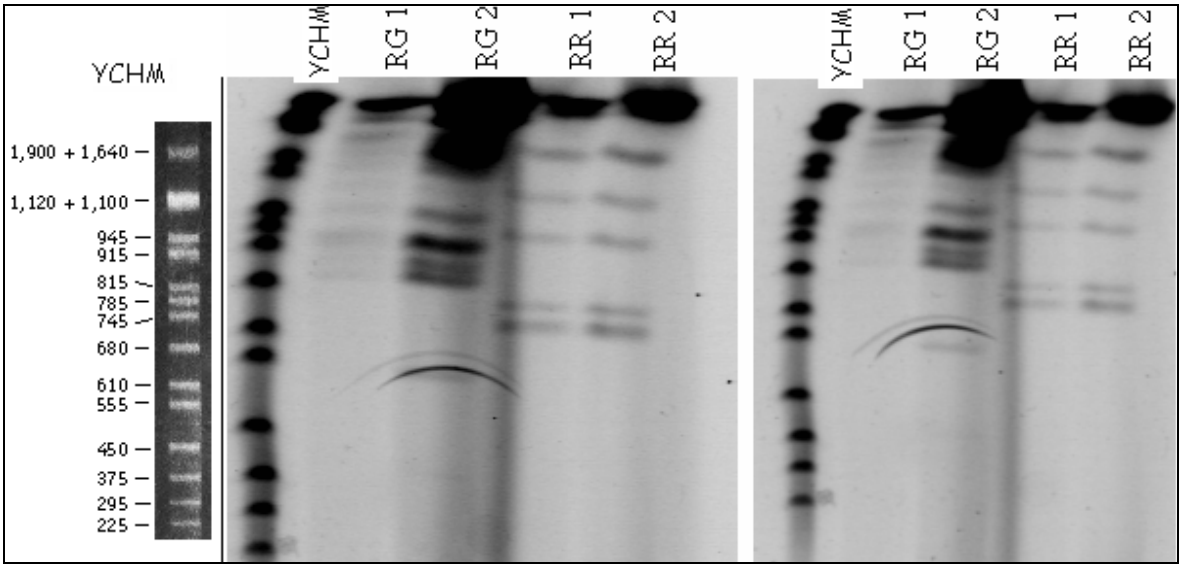


Figure 78: PFGE of carotenogenic yeast; optimilization of isolation of chromosomal DNA;
Conditions: 1.2% gel, 0.08M TBE, 162 V 70s/15h, 100s/10h, 120s/10h, 150s/15h;
Samples: YCHM – yeast chromosome marker; RG 1 – *Rhodotorula glutinis* 1; RG 2 - *Rhodotorula glutinis* 2;
RR 1 - *Rhodotorula rubra* 1; RR 2 - *Rhodotorula rubra* 2

The protocol with lower detergent and enzyme concentration (RG 2 - 24 plugs/7.5 % ME/5 mg proteinase K) was found to be more convenient for red yeast chromosomes isolation (See *Figure 78*). Higher detergent content can lead to DNA fragmentation and decrease in number of intact chromosomes.

- optimization of conditions for electrophoretic karyotyping of carotenogenic yeast

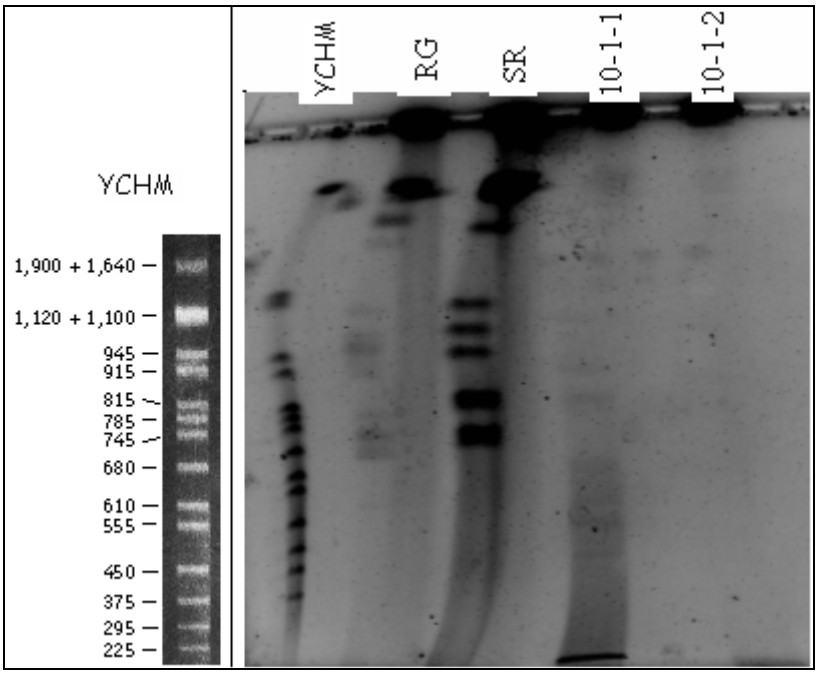


Figure 79: Pulsed Field Gel Electrophoresis of carotenogenic yeast;
Conditions: 1% gel, 1 M TBE, 100s/10h/162V, 120s/10h/162V, 150s/15h/162V, 200s/15h/135V, 250s/15h/108V, 300s/10h/108V
Samples: YCHM – yeast chromosome marker; RG - *Rhodotorula glutinis*; SR – *Sporobolomyces roseus*; 10-1-1 - *Cystofilobasidium capitatum* 10-1-1; 10-1-2 - *Cystofilobasidium capitatum* 10-1-2

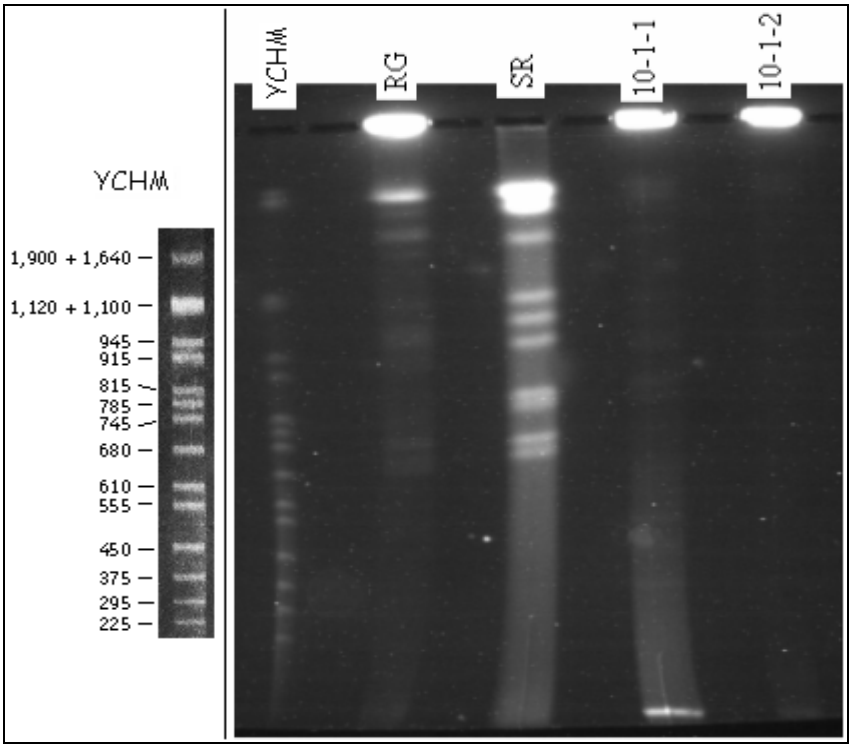


Figure 80: PFGE optimization for chromosome separation of carotenogenic yeast;
Conditions: 1% gel, 0.08M TBE, 100s/10h/162V, 120s/10h/162V, 150s/15h/162V, 200s/20h/162V; 250s/20h/108V;
Samples: YCHM – yeast chromosome marker; RG - *Rhodotorula glutinis*; SR – *Sporobolomyces roseus*; 10-1-1 - *Cystofilobasidium capitatum* 10-1-1; 10-1-2 - *Cystofilobasidium capitatum* 10-1-2

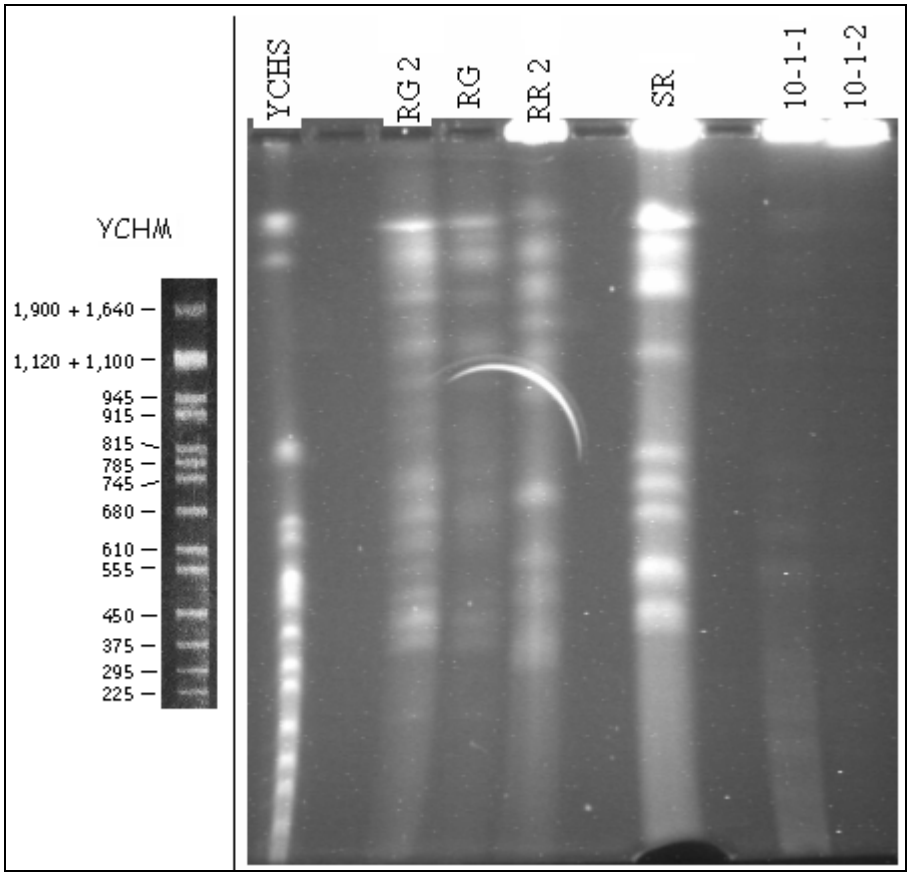


Figure 81: PFGE optimization for chromosome separation of carotenogenic yeast;
Conditions: 1% gel, 0.08M TBE, 100s/10h/162V, 120s/10h/162V, 150s/15h/162V, 200s/20h/162V, 250s/25h/108V, 300s/20h/108V;
Samples: YCHM – yeast chromosome marker; RG 2 - *Rhodotorula glutinis*; RG - *Rhodotorula glutinis*; RR 2 - *Rhodotorula rubra*; SR – *Sporobolomyces roseus*;
10-1-1 - *Cystofilobasidium capitatum* 10-1-1; 10-1-2 - *Cystofilobasidium capitatum* 10-1-2

After successful isolation of intact chromosomal DNA from *Rhodotorula sp.* and *Sporobolomyces sp.*, they were analyzed by pulsed field gel electrophoresis. In order to get electrophoratic karyotype of carotenogenic yeast with high resolution, various gel concentrations, TBE buffer concentration, voltage and switch times were tested. Values of mentioned parameters for PFGE analysis were chosen according to manufactor's instructions [84]. The following PFGE conditions: 1% gel, 0.08M TBE, 162V 100s/10h, 120s/10h, 150s/15h, 200s/20h, 108V 250s/25h, 300s/20h (See *Figure 81*) were found as optimal for separation of chromosomes of carotenogenic yeast *Rhodotorula* and *Sporobolomyces*. Our results indicate that red yeast *Rhodotorula glutinis* has about 15 chromosomes with sizes in range of 650 – 2000 bp, *Rhodotorula rubra* has about 13 chromosomes with sizes in range of 650 – 2000 bp and *Sporobolomyces roseus* has about 10 chromosomes with sizes in range of 680 – 2000 bp. By comparison of electrophoretic karyotypes of both *Rhodotorula sp.*, many similarities can be found. It confirms their genus conjunction.

When the protocol for intact chromosomal DNA isolation from carotenogenic yeast *Rhodotorula* and *Sporobolomyces* was established (See chapter 6.8.1. and 6.8.2.), it was applied on other red yeast, *Cystofilobasidium*. However, no bands were observed in the case of *Cystofilobasidium*. It seems that fragmentation of *Cystofilobasidium* DNA appears under these conditions.

6.8.3. Determination of DNA fragmentation

It was reported by Ribeiro [28] that chromosomal DNA from *S. cerevisiae* cells was fragmented during 200-min treatment with 10 mM hydrogen peroxide. During this process, the fraction of viable cells decreases from 100 to about 5–10%. The DNA in *S. cerevisiae* cells treated with 150 mM hydrogen peroxide remained intact for the 25 min necessary for a decrease from 100 to about 1% of surviving cells. This result shows that cell death is only associated with DNA fragmentation for treatment of relatively low intensity. At too high intensity of the treatment, the cell is presumed to die from complete breakdown (necrosis) before any PCD process can be initiated (See *Figure 82*).

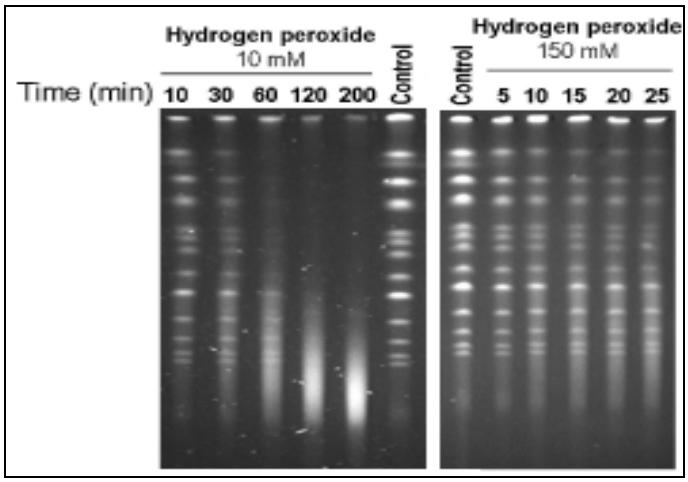


Figure 82: Genomic DNA from *S. cerevisiae* cells treated by H_2O_2 analyzed by PFGE [28]

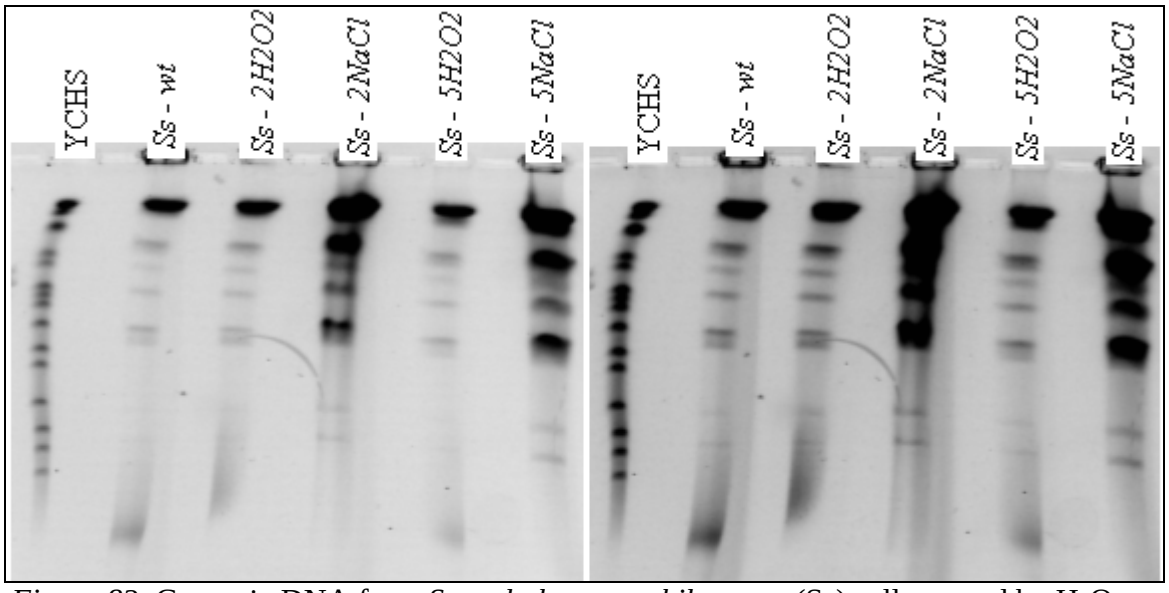


Figure 83: Genomic DNA from *Sporobolomyces shibatanus* (Ss) cells treated by H_2O_2 or NaCl analyzed by PFGE

Samples: YCHS – Yeast chromosome marker; *Ss – wt*: *S. shibatanus* wildtype; *Ss – 2H2O2*: *S. shibatanus* treated by 2 mM H_2O_2; *Ss – 2NaCl*: *S. shibatanus* treated by 2% NaCl; *Ss – 5H2O2*: *S. shibatanus* treated by 5 mM H_2O_2; *Ss – 5NaCl*: *S. shibatanus* treated by 5% NaCl;

In *Sporobolomyces shibatanus* cells treated with 2 – 5 mM H_2O_2 or 2 – 5% NaCl no DNA fragmentation was obtained (See *Figure 83*). These results confirm nontoxic effect of these concentrations of environmental stress on red yeasts.

6.9. Gene knockout

This part of PHD thesis was done within the frame of Erasmus intership in Max F. Perutz Laboratories, University Department at the Vienna Biocenter, Department of chromosomal biology, Vienna, Austria.

The availability of the yeast deletion set with target gene deletions makes it possible to identify, among all the nonessential genes in yeast, those genes whose products are required for specific cellular processes or contribute to stress resistance in a dose-dependent manner. Moreover function of these genes can be estimated. An efficient technique to knock out genes in fission yeast *Schizosaccharomyces pombe* was designed by Gregan group [52]. By this technique 47 out of 78 selected genes of *Sz. pombe* were successfully knock out.

6.9.1. Genomic sequence and identification of target genes

In this work, 47/78 genes from *Sz. pombe* was knocked out. For purposes of this work they were named as A1, 2 ... -12, B1, 2 ... -12, C1, 2 ... -12, D1, 2 ... -12, E1, 2 ... -12, F1, 2 ... -12 and G1,2 ... -6. In order to design homology regions of target gene, appropriate PCR primers for PCR amplification of homology regions, restriction sites and restriction profil (gel preview), checking primers, etc., gene sequence need to be known. DNA sequences of mentioned genes, with their basic characteristics can be found in *Sz. pombe* genome database. Different softwares are available to design cloning experiments. JellyFish (Biowire) (See *Figure 85*) and VectorNT (Invitrogen) are the most widely known. Moreover, the deletion flowchart for majority of genes from *Sz. pombe* can be found at http://mendel.imp.ac.at/Pombe_deletion/ (See *Figure 86*).

6.9.2. Isolation of gDNA from *Sz. pombe* and PCR amplification of homology regions

The genomic DNA from *Sz. pombe* cells was isolated as described in Matrials and Methods (See chapter 5.14.1.) and used for amplification of homology regions of target gene (See *Figure 84*). The size of PCR products assigned according to 1 kb DNA ladder was compared with expected size and the correct ones were chosen for next work.

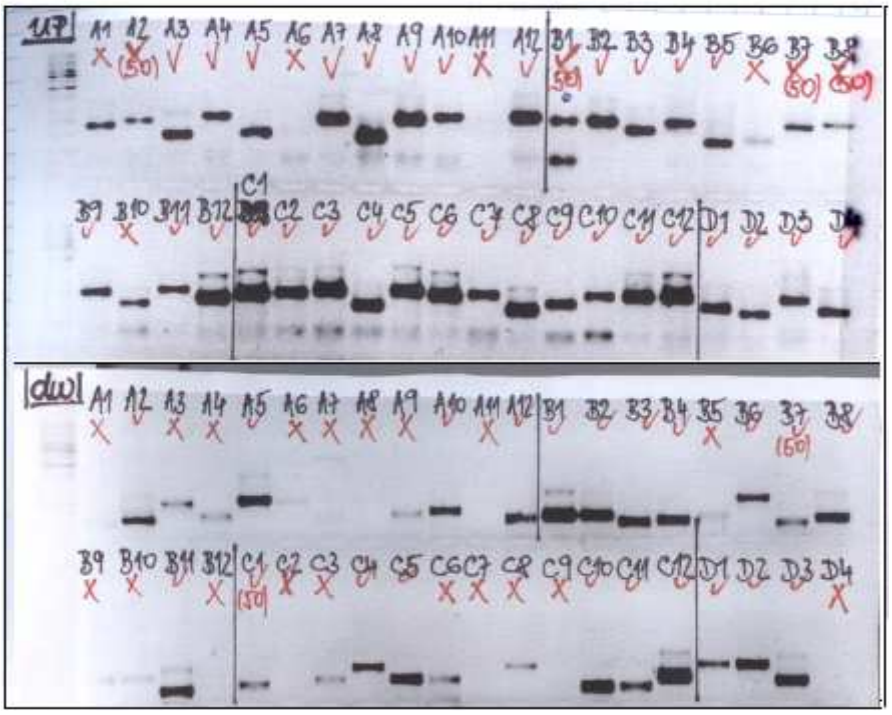


Figure 84: PCR amplification of homology regions

Figure 85: Nucleotide seguence of mug166 in Jellyfish SW

Figure 86: Basic characteristics important for gene SEC13 knock out

6.9.3. Insert preparation

Each amplified homology region is carring two restriction sites (EnzA/Enz1 – up part, EnzB/Enz1 – dw part) (See *Figure 18*). Amplified homology regions with inserted restriction sites were digested by appropriate endonucleases, ligated together as described in Materials and Methods (See chapter 5.14.3.) and resulting heterodimer was cut and extracted from an agarose gel (See *Figure 87*) and cloned into the vector.

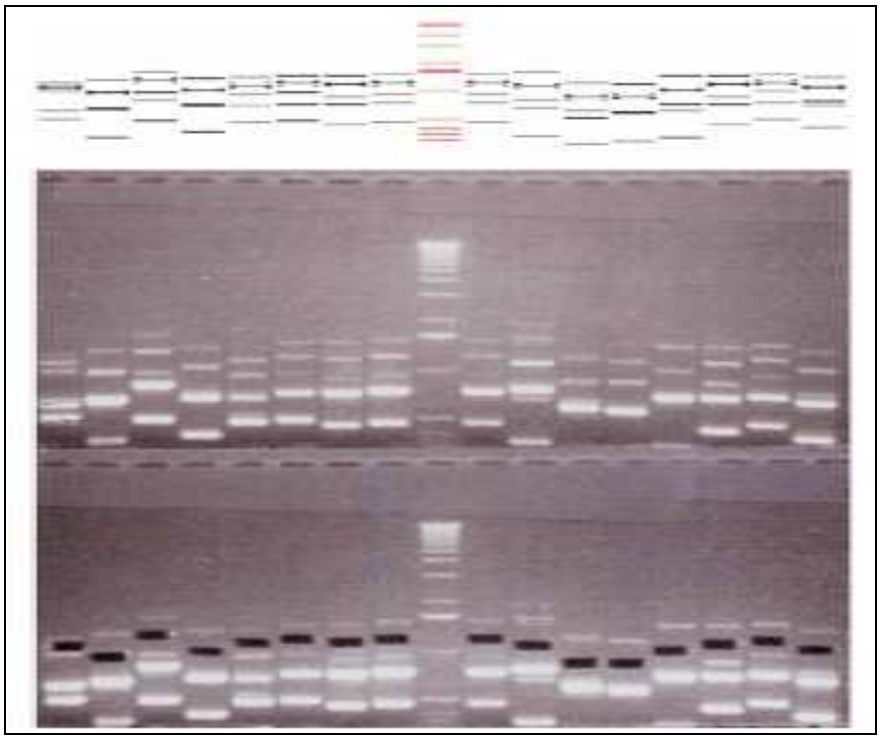


Figure 87: Comparison of ligated homology regions with the gel preview at http://mendel.imp.ac.at/Pombe_deletion/ (See *Figure 86*). Bands highlighted on the gel preview represent correctly ligated homology regions. These bands are cut out of the gel and cloned into the vector [52]

6.9.4. Vector preparation

Cloning vector pCloneKan1 (See *Figure 88*) conferring resistance to genetecine was used. The geneticine resistance gene was chosen because it has so far not been widely used in fission yeast, which can facilitate the generation of deletions in most of the existing fission yeast strain. Nucleotide sequence of pCloneKan1 can be found in GenBank.

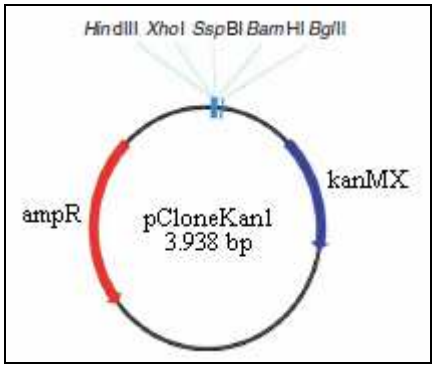


Figure 88: Maps of cloning vector pCloneKan1. Restriction sites used for cloning of the homology regions are indicated

6.9.5. Cloning of homology regions into vector and amplification of new plasmid in *Escherichia coli*

Prepared insert digested by appropriate restriction enzymes (EnzA/EnzB) (See chapter 6.9.3.) was cloned into vector digested by the same restriction enzymes (See chapter 6.9.4.) and amplified in *E. coli* cells (See *Figure 89*).

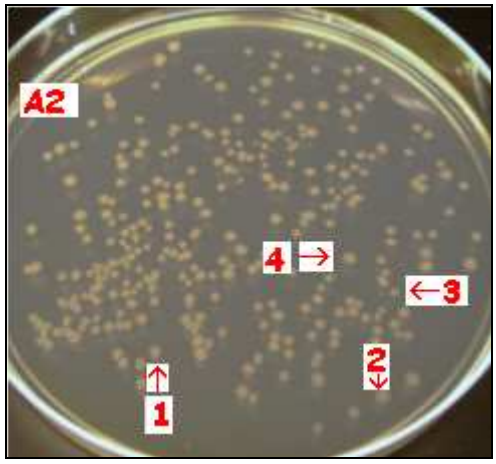


Figure 89: *E. coli* transformants

Positive transformants were selected by PCR using checking primers (See *Figure 90*) so that size of PCR product was compared with expected values (See *Figure 86*).

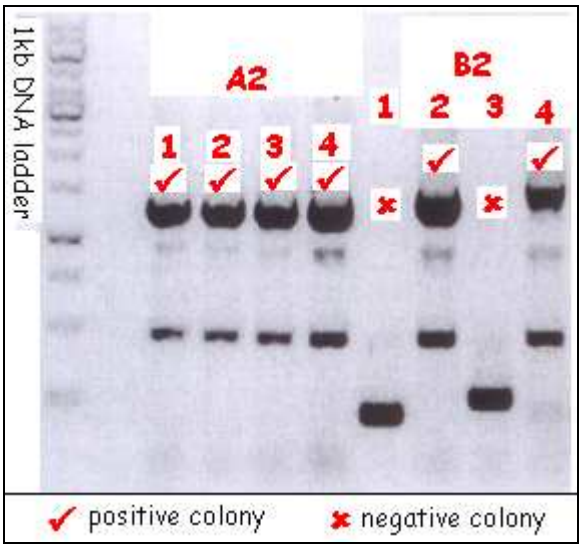


Figure 90: PCR checking of *E. coli* transformants

6.9.6. *Sz. pombe* transformation – deletion of definite gene

Construct (vector containing intended insert) isolated from positive *E. coli* transformants was linearized (digested by Enz1) (See *Figure 91*) and transformed into *Sz. pombe* cells as described in Materials and Methods (See chapter 5.14.7.). Transformants with deleted genes were selected based on geneticine resistance and verified by PCR using designed yeast checking primers (See *Figure 92*).

Figure 91: Construct checking and linearization (restriction by Enz1)

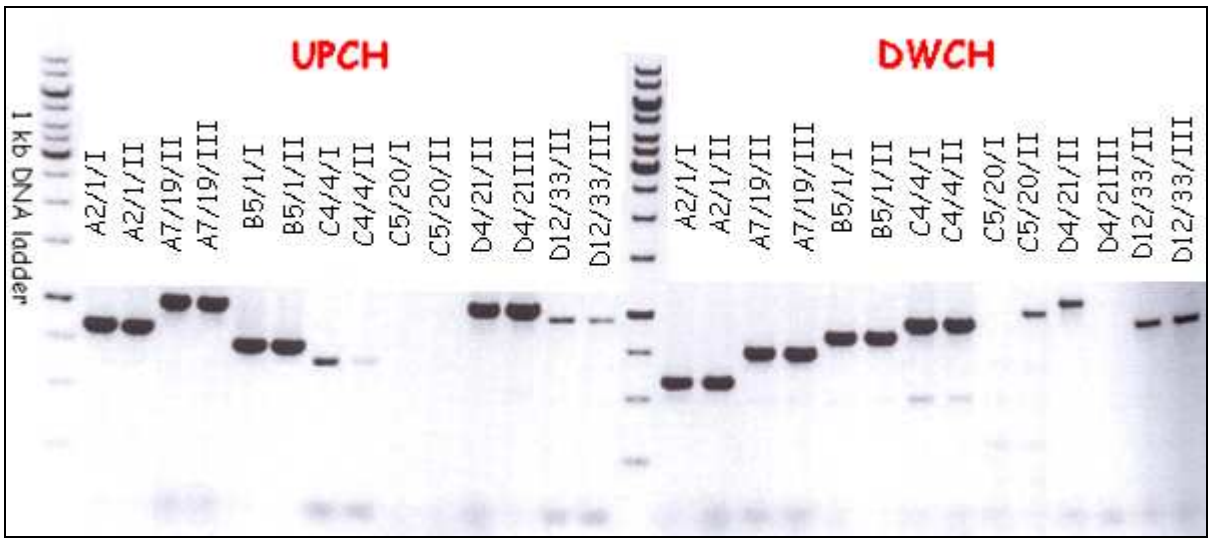


Figure 92: Checking of yeast transformants

Described technique was based on knockout constructs that contain regions homologous to the target gene cloned into vectors carrying dominant drug-resistance markers. By this technique 47 out of 78 selected genes of *Sz. pombe* were successfully knock out. A strategy for cloning knockout constructs for all predicted fission yeast genes is available in a form of a searchable database http://mendel.imp.ac.at /Pombe_deletion. This technique was validated in a screen where novel genes required for chromosome segregation during meiosis were identified [85].

7. CONCLUSIONS

- ✓ Yeast is, due to its physiological properties, widely used in the food, feed, chemical and pharmaceutical industries for production of various valuable compounds. For instance, red yeast is well known producer of carotenoids which are significant because of their activity as vitamin A precursors, colorants, antioxidants and possible tumor-inhibiting agents. Biological sources of carotenoids receive major focus nowadays because of the stringent rules and regulations applied to chemically synthesized/purified pigments. Compared with the extraction from vegetables, the microbial production of carotenoids is of paramount interest, mainly because of the problems of seasonal and geographic variability in the production and marketing of several of the colorants of plant origin. Moreover, red yeast is a rich source of fats, proteins and vitamins and can be incorporated in feeds to enhance the nutritional value and prevent fungal contamination. One limitation impacting the industrial utility of carotenogenic yeast has been complicated extraction of carotenoids, due to the yeast's thick cell wall and questionable analysis and identification of individual carotenoids, due to their structural similarities and light and temperature sensitivity.

- ✓ The biotechnological industry has developed different means of pigment liberation by the yeast including optimization of drying conditions, mechanical breakage, microwave treatment and enzyme treatment. Accurate identification and quantitation of all analytes present in a sample requires their complete chromatographic resolution. High-performance liquid chromatography is regarded as the preferred method for the separation of carotenoids found in biological matrices. In this work, satisfactory separation of carotenoid pigments extracted from red yeast was carried out using a C18 reversed-phase column and methanol as the solvent system at a flow rate of 1 ml/min. Mass spectrometric and tandem mass spectrometric analyses, which provide molecular weight and characteristic fragmentation patterns, may provide final confirmation of individual carotenoids when used in conjunction with retention and spectral characteristics. In the last years, positive ion electrospray LC-MS was shown to be useful for the analysis of carotenes. In this study, the *β*-carotene was determined as the major carotenoid pigment in carotenogenic yeasts.

- ✓ The other very important limitation involved in the practical exploitation of yeast is the high cost of microbial production. The production cost could be reduced by increasing yields of product, as well as using less expensive substrates. There is a need to improve fermentation strategies such that the inracellular accumulation of carotenoid from yeast is feasible on an industrial scale. Biomass and metabolites production by red yeast is highly variable and can be influenced by cultivation conditions (light, temperature, pH, aeration...). Different approaches for improving the production properties of the yeast strains, such as environmental stress, mutagenesis or genetic modification, have been studied and optimized. The other possibility for production cost reduction is using various low-cost materials as carbon or nitrogen source. In this work, the potential of several waste materials (whey, potato mass, apple mass and various cereals) as substrates for carotenoid and ergosterol production by some yeast strains belonging to the genus *Rhodotorula* and *Sporobolomyces* were examined. Our results demonstrated that potato extract or lyophillized whey non-processed as well as deproteinated could be used as a suitable carbohydrate source for carotenogenesis and ergosterol production in *R. glutinis*. Maximal β-carotene and

ergosterol yields in the yeast *Rhodotorula rubra* were obtained in media with non-processed lyophilized whey added into inoculation and production media and in media with potato extract also added into both media. Lyophilized whey non-processed or deproteinated and potato extract were found to be the most suitable for carotenogenesis or ergosterol production also in the yeast *Sporobolomyces roseus.*

- ✓ As mentioned above, environmental stress was reported to induce carotenoid production as part of red yeast stress response. On the other hand, the environmental stress can damage biological molecules and under nonphysiological doses lead to cell death. In other words, under environmental stress cells posses altered phenotype biotechnologically significant and/or undesirable in a dose-dependent manner. Phenotypic profiling of the environmental stress responses demonstrates genetic susceptibility of yeast to environmental stress. Measurements of cell viability and growth provide versatile and sensitive assays for characterization of cytotoxic effect of environmental stress. Yeast cells exhibit a graded concentration-dependent response to chemically induced stress: continued growth, cellular adaptation, checkpoint arrest/growth delay, apoptosis, and necrosis. Our results demonstrate that low concentrations of oxidative and osmotic stress, which can under specific conditions induce carotenogenesis, have no significant effect on yeast growth. Morphological changes are visible indicators of microbial adaptability to environment. Microscopic observation of morphology of yeast exposed to environmental stress can provide futher information on cytotoxicity of environmental stress. Red yeast cultivated under osmotic and oxidative stress or on various waste substrates shows no significant differences in cell morphology when compared with yeast cultivated in conventional glucose medium under optimal conditions. Typical ellipsoidal shaped cells were mainly observed. In some cases, thicker cell wall was formed what could indicate higher accumulation of carotenoid present in cell wall. Under higher concentrations of osmotic stress shrunk cells with wall damage were appeared. Also these results confirm that low concentrations of environmental stress have no cytotoxic effect on yeast cell.

- ✓ ROS accumulation is next indicator of severity of environmental stress. Higher ROS abundance in yeast cells can result from programmed cell death. Generation of $O_2^{-}\cdot$ was measured by red fluorescent cell staining due to oxidation of DHE by $O_2^{-}\cdot$. No $O_2^{-}\cdot$ was produced in either adapted or in control cells. $O_2^{-}\cdot$ formation was observed only in cells treated with 100 mM H_2O_2. Production of H_2O_2 occurred in cells cultivated under strong oxidatve stress (100 mM H_2O_2), as shown by the presence of intense green fluorescence stained cells, due to H_2O_2-dependent oxidation of H_2DCF to DCF. In control cells no H_2O_2 generation was observed, thus suggesting that its production is specifically caused by H_2O_2 treatment. Under conditions of osmotic stress, maximal $O_2^{-}\cdot$ and H_2O_2 generation was observed in cells treated with 10 % NaCl. It suggests that cells of *Rhodotorula glutinis* can adapt to lower concentrations of osmotic stress and so these concentrations can be used to stimulate carotenoid production.

- ✓ The other important marker of environmental stress toxicity is chromosomal DNA fragmentation. Pulsed field gel electrophoresis of chromosomal DNA from cells exposed to environmetal stress can reveal DNA breakdown into fragments of several hundred kilobases. Studies on electrophoretic karotype of yeast are limited to organisms from which high quality protoplasts can be prepared in large numbers.

Protoplasts preparation from red yeasts is very difficult due to unusual cell wall character. Different types of lytic enzymes (glycosidases and glucuronidases) and combination of several procedures is necessary to use. Protoplasts in *Rhodotorula* sp. and *Sporobolomyces* sp. were formed and can be osmotically disrupted after substantially longer period (7-9 hours) than in *Sacharomyces cerevisiae* (30 min) and *Phaffia rhodozyma* cells (2 hours). Isolation of chromosomes in agarose blocks with lower detergent and enzyme concentration (24 plugs/7.5 % ME/5 mg proteinase K) was found to be the most convenient protocol for red yeast intact chromosomes isolation. Intact chromosomal DNA extracted from red yeast was then analyzed by pulsed field gel electrophoresis. The following PFGE conditions: 1% gel, 0.08M TBE, 162V 100s/10h, 120s/10h, 150s/15h, 200s/20h, 108V 250s/25h, 300s/20h were found as optimal for separation of chromosomes of carotenogenic yeast *Rhodotorula* and *Sporobolomyces*. Our results indicate that red yeast *Rhodotorula glutinis* has about 15 chromosomes with sizes in range of kbp, *Rhodotorula rubra* has about 13 chromosomes with sizes in range of kbp and *Sporobolomyces roseus* has about 10 chromosomes with sizes in range of kbp. By comparison of electrophoretic karyotypes of both *Rhodotorula sp.*, many similarities can be found. Next DNA integrity was studied. In *Sporobolomyces shibatanus* cells treated with $2-5$ mM H_2O_2 or $2-5$% NaCl, no DNA fragmentation was obtained. These results confirm nontoxic effect of these concentrations of environmental stress on red yeasts.

- ✓ One important aspect of environmental stress response of yeast is genome reorganization. The availability of the yeast deletion set with target gene deletions makes it possible to identify, among all the nonessential genes in yeast, those genes whose products are required for specific cellular processes or contribute to stress resistance in a dose-dependent manner. Moreover function of these genes can be estimated. An efficient technique to knock out genes in fission yeast *Schizosaccharomyces pombe* was designed by Gregan group. This technique is based on knockout constructs that contain regions homologous to the target gene cloned into vectors carrying dominant drug-resistance markers. By this technique 70 from last 100 nondeleted genes of *Sz. pombe* were successfully knock out. A strategy for cloning knockout constructs for all predicted fission yeast genes is available in a form of a searchable database http://mendel.imp.ac.at/Pombe_deletion. This technique was validated in a screen where novel genes required for chromosome segregation during meiosis were identified. According to this protocol, a strategy to knock out genes in red yeast could be designed. The functional analysis of the carotenogenic genes of yeast can facilitate the study of the effect of their overexpression on carotenoid biosynthesis and improve production properties of yeast strain.

8. REFERENCES

1. A. TALARO and K. P. TALARO: *Foundations in Microbiology*. 2001, Pasadena: McGraw-Hill. 889 p. ISBN 0-07-248864-6
2. G. M. WALKER: *Yeast Physiology and Biotechnology*. 1998, West Sussex, England: John Willey & Sons Ltd. 362 p. ISBN 0-471-964468
3. A. KOCKOVÁ-KRATOCHVÍLOVÁ: *Yeasts and Yeast-like Organisms*. 1990, Weiheim: VCH Verlagsgesellschaft. 528 p. ISBN 3-527-26162-1
4. H. FELDMANN. *Yeast Molecular Biology*. [online] 2005 [cited 26. 6. 2009]; Available from: http://biochemie.web.med.uni-muenchen.de/Yeast_Biol/.
5. J. MANN: *Secondary Metabolism*. Second edition. 1990, USA: Oxford University Press. 390 p. ISBN 0198555296
6. *Introduction to Flow Cytometry: A Learning Guide*. 2000, BD Biosciences: San Jose, California.
7. A. P. J. MIDDELBERG: *Process-scale disruption of microorganisms.* Biotechnology Advances, 1995. **13**(3): p. 491-551. ISSN 0734-9750
8. M. J. WAITES, N. L. MORGAN, J. S. ROCKEY, and G. HIGTON: *Industrial Microbiology: An Introduction*. 2001, Oxford: Wiley-Blackwellp. ISBN 978-0-632-05307-0
9. THERMO FISHER SCIENTIFIC. *Overview of Cell Lysis and Protein Extraction.* [online] 2009 [cited 24. 8. 2009]; Available from: http://www.piercenet.com/browse.cfm?fldID=FA97D803-6953-48E4-A7BD-6947D35FE83B&Format=Print.
10. QIAGEN. *Genomic DNA Purification.* [online] [cited 8. 8. 2009]; Available from: http://www1.qiagen.com/literature/brochures/Gen_DNA_Pur/1019469_BROS_DNYTi_p10_13.pdf.
11. J. L. LOPEZ: *Two-dimensional electrophoresis in proteome expression analysis.* Journal of Chromatography B, 2007. **849**(1-2): p. 190-202. ISSN 1570-0232
12. B. ALBERTS, D. BRAY, K. HOPKIN, A. JOHNSON, J. LEWIS, M. RAFF, K. ROBERTS, and P. WALTER: *Essential Cell Biology*. Second edition. 2004, New York: Garland Science. 740 p. ISBN 0-8153-3480-X
13. S. L. FORSBURG: *The yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe: models for cell biology research.* Gravitational and Space Biology Bulletin: Publication of the American Society for Gravitational and Space Biology, 2005. **18**(2): p. 3-9. ISSN 1089-988X (print)
14. W. XIAO: *Yeast Protocols (Methods in Molecular Biology).* Second edition. 2005, Totowa, New Jersey: Humana Press. 392 p. ISBN 1588294374
15. S. HOHMANN and W. H. MAGER: *Yeast Stress Responses*. 2003, Berlin: Springer-Verlag. 389 p. ISBN 3-540-43926-9
16. R. EGEL: *The Molecular Biology of Schizosaccharomyces pombe: Genetics, Genomics and Beyond*. First edition. 2007, Verlag, Berlin, Heidelberg, New York: Springer. 450 p. ISBN 3540006931
17. C. ROSA and G. PETER: *Biodiversity and Ecophysiology of Yeasts (The Yeast Handbook).* 2005, Berlin, Heidelberg, New York: Springer. 580 p. ISBN 3540261001
18. J. R. LEPOCK, E. E. BITTAR, and J. S. WILLIS: *Protein Denaturation During Heat Shock*. Advances in Molecular and Cell Biology. Volume 19. 1997: Elsevier. 223-259 p. ISBN 1569-2558
19. P. W. PIPER: *Molecular events associated with acquisition of heat tolerance by the yeast Saccharomyces cerevisiae.* FEMS Microbiology Reviews, 1993. **11**(4): p. 339-355. ISSN 0168-6445

20. J. R. LEPOCK: *Measurement of protein stability and protein denaturation in cells using differential scanning calorimetry.* Methods, 2005. **35**(2): p. 117-125. ISSN 1046-2023
21. S. W. RYTER, H. P. KIM, A. HOETZEL, J. W. PARK, K. NAKAHIRA, X. WANG, and A. M. K. CHOI: *Mechanisms of cell death in oxidative stress.* Antioxidants & Redox Signaling, 2007. **9**(1): p. 49-89. ISSN 1523-0864
22. G. G. PERRONE, S. X. TAN, and I. W. DAWES: *Reactive oxygen species and yeast apoptosis.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2008. **1783**(7): p. 1354-1368. ISSN 0167-4889
23. B. HALLIWELL: *Free radicals and metal ions in health and disease.* The Proceedings of the Nutrition Society, 1987. **46**(1): p. 13-26. ISSN 0029-6651
24. B. HALLIWELL and J. M. GUTTERIDGE: *Lipid peroxidation, oxygen radicals, cell damage, and antioxidant therapy.* Lancet, 1984. **1**(8391): p. 1396-1397. ISSN 0140-6736 (print), 1474-547X (electronic)
25. B. HALLIWELL and O. I. ARUOM: *DNA damage by oxygen-derived species: its mechanism and measurement in mammalian systems.* FEBS Letters, 1991. **281**(1-2): p. 9-19. ISSN 0014-5793
26. R. L. LEVINE, J. A. WILLIAMS, E. P. STADTMAN, E. SHACTER, and P. LESTER: *Carbonyl assays for determination of oxidatively modified proteins*. Methods in Enzymology. Volume 233. 1994: Academic Press. 346-357 p. ISBN 0076-6879
27. E. CABISCOL, E. PIULATS, P. ECHAVE, E. HERRERO, and J. ROS: *Oxidative stress promotes specific protein damage in Saccharomyces cerevisiae.* The Journal of Biological Chemistry, 2000. **275**(35): p. 27393 – 27398. ISSN 0021-9258 (print), 1083-351X (electronic)
28. G. F. RIBEIRO, M. CORTE-REAL, and B. JOHANSSON: *Characterization of DNA damage in yeast apoptosis induced by hydrogen peroxide, acetic acid, and hyperosmotic shock* Molecular Biology of the Cell, 2006. **17**(10): p. 4584-4591. ISSN 1939-4586
29. S. HOHMANN: *Osmotic stress signaling and osmoadaptation in yeasts.* Microbiology and Molecular Biology Reviews, 2002. **66**(2): p. 300 - 372. ISSN 1098-5557
30. F. POSAS, J. R. CHAMBERS, J. A. HEYMAN, J. P. HOEFFLER, E. NADAL, and J. ARINO: *The transcriptional response of yeast to saline stress.* The Journal of Biological Chemistry, 2000. **275**(23): p. 17249-17255. ISSN 0021-9258 (print), 1083-351X (electronic)
31. A. P. GASCH and M. WERNER-WASHBURNE: *The genomics of yeast responses to environmental stress and starvation.* Functional & Integrative Genomics, 2002. **2**(4-5): p. 181-192. ISSN 1438-793X (print), 1438-7948 (electronic)
32. G. M. GADD and A. J. GRIFFITHS: *Microorganisms and heavy metal toxicity.* Microbial Ecology, 1977. **4**(4): p. 303-317. ISSN 0095-3628 (print), 1432-184X (electronic)
33. A. P. GASCH, M. HUANG, S. METZNER, D. BOTSTEIN, S. J. ELLEDGE, and P. O. BROWN: *Genomic expression responses to DNA-damaging agents and the regulatory role of the yeast ATR homolog Mec1p.* Molecular Biology of the Cell, 2001. **12**(10): p. 2987-3003. ISSN 1059-1524 (print), 1939-4586 (electronic)
34. D. CLARK: *Molecular Biology*. 2005, LONDON: Elsevier Academic Press. 816 p. ISBN 0-12-175551-7
35. A. E. YOUSEF and V. K. JUNEJA: *Microbial Stress Adaptation and Food Safety*. 2002, Florida: CRC Press. 384 p. p. ISBN 1566769124

36. F. ESTRUCH: *Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast.* FEMS Microbiology Reviews, 2000. **24**(4): p. 469-486. ISSN 0168-6445
37. H. F. GILBERT: *Basic Concepts in Biochemistry: A Student's Survival Guide.* Second edition. 1999, New York: McGraw-Hill Medical. 331 p. ISBN 0071356576
38. A. P. GASCH, P. T. SPELLMAN, C. M. KAO, O. CARMEL-HAREL, M. B. EISEN, G. STORZ, D. BOTSTEIN, and P. O. BROWN: *Genomic expression programs in the response of yeast cells to environmental changes.* Molecular Biology of the Cell, 2000. **11**(12): p. 4241-4257. ISSN 1059-1524 (print), 1939-4586 (electronic)
39. M. T. MARTINEZ-PASTOR, G. MARCHLER, C. SCHULLER, A. MARCHLER-BAUER, H. RUIS, and F. ESTRUCH: *The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE).* The EMBO Journal, 1996. **15**(9): p. 2227-2235. ISSN: 0261-4189 (print), 1460-2075 (electronic)
40. W. GORNER, E. DURCHSCHLAG, M. T. MARTINEZ-PASTOR, F. ESTRUCH, G. AMMERER, B. HAMILTON, H. RUIS, and C. SCHULLER: *Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity.* Genes & Development, 1997. **12**(4): p. 586-597. ISSN: 0890-9369 (print), 1549-5477 (electronic)
41. A. K. PEARCE and T. C. HUMPHREY: *Integrating stress-response and cell-cycle checkpoint pathways.* Trends in Cell Biology, 2001. **11**(10): p. 426-433. ISSN 0962-8924
42. P. A. DELLEYA and M. N. HALLA: *Cell wall stress depolarizes cell growth via hyperactivation of RHO1* The Journal of Cell Biology, 1999. **147**(1): p. 163-174. ISSN 0021-9525 (print), 1540-8140 (electronic)
43. K. A. MORANO, P. C. C. LIU, and D. J. THIELE: *Protein chaperones and the heat shock response in Saccharomyces cerevisiae.* Current Opinion in Microbiology, 1998. **1**(2): p. 197-203. ISSN 1369-5274
44. J. G. KIANG and G. C. TSOKOS: *Heat Shock Protein 70 kDa: Molecular Biology, Biochemistry, and Physiology.* Pharmacology & Therapeutics, 1998. **80**(2): p. 183-201. ISSN 0163-7258
45. B. PANARETOU and CH. ZHAI: *The heat shock proteins: their roles as multi-component machines for protein folding.* Fungal Biology Reviews, 2009. **22**(3-4): p. 110-119. ISSN 1749-4613
46. D. J. JAMIESON: *Oxidative stress responses of the yeast Saccharomyces cerevisiae.* Yeast, 1998. **14**(16): p. 1511-1527. ISSN 0749-503X
47. P. MORADAS-FERREIRA, V. COSTA, P. PIPER, and W. MAGER: *The molecular defences against reactive oxygen species in yeast.* Molecular Microbiology, 1996. **19**(4): p. 651-658. ISSN 0950-382X
48. R. L. LEVINE, L. MOSONI, B. S. BERLETT, and E. R. STADTMAN: *Methionine residues as endogenous antioxidants in proteins.* Proceedings of the National Academy of Sciences of the United States of America, 1996. **93**(26): p. 15036–15040. ISSN 1091-6490
49. J. FRANCOIS and J. L. PARROU: *Reserve carbohydrates metabolism in the yeast Saccharomyces cerevisiae.* FEMS Microbiology Reviews, 2001. **25**(1): p. 125-145. ISSN 0168-6445 (print), 1574-6976 (electronic)
50. C. A. MICHELS: *Genetic Techniques for Biological Research. A Case Study Approach*. 2002, New York: John Wiley & Sons, Ltd. 254 p. ISBN 0-471-89921-6 (Hardback); 0-470-84662-3 (Electronic)

51. T. HIRASAWA, K. YOSHIKAWA, Y. NAKAKURA, K. NAGAHISA, CH. FURUSAWA, Y. KATAKURA, H. SHIMIZU, and S. SHIOYA: *Identification of target genes conferring ethanol stress tolerance to Saccharomyces cerevisiae based on DNA microarray data analysis.* Journal of Biotechnology, 2007. **131**(1): p. 34-44. ISSN 0168-1656
52. J. GREGAN, P. K. RABITSCH, C. RUMPF, M. NOVATCHKOVA, A. SCHLEIFFER, and K. NASMYTH: *High-throughput knockout screen in fission yeast.* Nature Protocols, 2006. **1**: p. 2457 - 2464. ISSN 1754-2189 (print), 1750-2799 (electronic)
53. D. LIBKIND, R. SOMMARUGA, H. ZAGARESE, and M. VAN BROOCK: *Mycosporines in carotenogenic yeasts.* Systematic and Applied Microbiology, 2005. **28**(8): p. 749-754. ISSN 0723-2020
54. G. S. DE HOOG, J. GUARRO, J. GENE, and M. J. FIGUERAS: *Atlas of Clinical Fungi.* Second edition. 2001, Washington: ASM Pressp. ISBN 9070351439
55. V. PERRIER, E. DUBREUCQ, and P. GALZY: *Fatty acid and carotenoid composition of Rhodotorula strains.* Archives of Microbiology, 1995. **164**(3): p. 173-179. ISSN 0302-8933 (print), 1432-072X (electronic)
56. G. BRITTON, S. LIAAEN-JENSEN, and H. PFANDER: *Carotenoids, Volume 4: Natural Functions*. 2008, Basel – Boston - Berlin: Birkhäuser Verlag. 370 p. ISBN 978-3-7643-7498-3
57. G. A. ARMSTRONG and J. E. HEARST: *Carotenoids 2: Genetics and molecular biology of carotenoid pigment biosynthesis.* The FASEB Journal, 1996. **10**(2): p. 228-237. ISSN 0892-6638 (print), 1530-6860 (electronic)
58. T. W. GOODWIN: *Biochemistry of the Carotenoids*. 1980, Berlin: Springerp. ISBN 978-0412216909
59. P. DAVOLI and R. W. S. WEBER: *Carotenoid pigments from the red mirror yeast, Sporobolomyces roseus.* Mycologist, 2002. **16**(3): p. 102-108. ISSN 0269-915X
60. T. W. GOODWIN: *Biosynthesis of carotenoids: an overview*. Methods in Enzymology, 1993. **214**: p. 330-340. ISSN 0076-6879
61. G. I. FRENGOVA and D. M. BESHKOVA: *Carotenoids from Rhodotorula and Phaffia: yeasts of biotechnological importance.* Journal of Industrial Microbiology & Biotechnology, 2009. **36**(2): p. 163-180. ISSN 1367-5435 (print), 1476-5535 (electronic)
62. R. EDGE, D. J. MCGARVEY, and T. G. TRUSCOTT: *The carotenoids as anti-oxidants — a review*. Journal of Photochemistry and Photobiology B: Biology, 1997. **41**(3): p. 189-200. ISSN 1011-1344
63. P. BHOSALE: *Environmental and cultural stimulants in the production of carotenoids from microorganisms.* Applied Microbiology and Biotechnology, 2003. **63**(4): p. 351-361. ISSN 0175-7598 (print), 1432-0614 (electronic)
64. H. T. GORDON and J. C. BAUERNFEIND: *Carotenoids as food colorants.* CRC Critical Reviews in Food Science and Nutrition, 1982. **18**(1): p. 59-97. ISSN 0099-0248
65. H. J. NELIS and A. P. DE LEENHEER: *Microbial sources of carotenoid pigments used in foods and feeds.* Journal of Applied Microbiology, 2008. **70**(3): p. 181 - 191. ISSN 1364-5072
66. R. B. VAN BREEMEN: *Electrospray liquid chromatography-mass spectrometry of carotenoids.* Analytical Chemistry, 1995. **67**(13): p. 2004-2009. ISSN 0003-2700 (print), 1520-6882 (electronic)

67. R. B. VAN BREEMEN: *Liquid chromatography/mass spectrometry of carotenoids.* Pure and Applied Chemistry, 1997. **69**(10): p. 2061-2066. ISSN 0033-4545 (print), 1365-3075 (electronic)
68. D. B. RODRIGUEZ-AMAYA: *A Guide to Carotenoid Analysis in Foods*. 2001, Washington: ILSI PRESS. 64 p. ISBN 1-57881-072-8
69. M. CARERI, L. ELVIRI, and A. MANGIA: *Liquid chromatography-electrospray mass spectrometry of [beta]-carotene and xanthophylls: Validation of the analytical method.* Journal of Chromatography A, 1999. **854**(1-2): p. 233-244. ISSN 0021-9673
70. R. B. VAN BREEMEN, CH. R. HUANG, Y. TAN, L. C. SANDER, and A. B. SCHILLING: *Liquid chromatography/mass spectrometry of carotenoids using atmospheric pressure chemical ionization.* Journal of Mass Spectrometry, 1997. **31**(9): p. 975-981. ISSN 1076-5174
71. D. LIBKIND, M. GADANHO, M. VAN BROOCK, and J. P. SAMPAIO: *Studies on the heterogeneity of the carotenogenic yeast Rhodotorula mucilaginosa from Patagonia, Argentina.* Journal of Basic Microbiology, 2008. **48**(2): p. 93-98. ISSN 0233-111X (print), 1521-4028 (electronic)
72. B. V. LATHA, K. JEEVARATNAM, H. S. MURALI, and K. S. MANJA: *Influence of growth factors on carotenoid pigmentation of Rhodotorula glutinis DFR-PDY from natural source.* Indian Journal of Biotechnology, 2005. **4**: p. 353-357. ISSN 0972-5849
73. A. HALIENOVA, I. MAROVA, M. CARNECKA, H. KONECNA, V. HANUSOVA, and V. HEZINOVA: *Proteome and metabolome changes in red yeasts grown under exogenous stress.* Journal of Biotechnology, 2007. **131**(2, Supplement 1): p. S202-S202. ISSN 0168-1656
74. I. MAROVA, E. BREIEROVA, R. KOCI, Z. FRIEDL, B. SLOVAK, and J. POKORNA: *Influence of exogenous stress factors on production of carotenoids by some strains of carotenogenic yeasts.* Annals of Microbiology, 2004. **54**(1): p. 73-85. ISSN 1590-4261
75. P. BUZZINI and A. MARTINI: *Production of carotenoids by strains of Rhodotorula glutinis cultured in raw materials of agro-industrial origin.* Bioresource Technology, 2000. **71**(1): p. 41-44. ISSN 0960-8524
76. Z. AKSU and A. T. EREN: *Carotenoids production by the yeast Rhodotorula mucilaginosa: Use of agricultural wastes as a carbon source.* Process Biochemistry, 2005. **40**(9): p. 2985-2991. ISSN 1359-5113
77. Z. AKSU and A. T. EREN: *Production of carotenoids by the isolated yeast of Rhodotorula glutinis.* Biochemical Engineering Journal, 2007. **35**(2): p. 107-113. ISSN 1369-703X
78. J. TINOI, N. RAKARIYATHAM, and R. L. DEMING: *Simplex optimization of carotenoid production by Rhodotorula glutinis using hydrolyzed mung bean waste flour as substrate.* Process Biochemistry, 2005. **40**(7): p. 2551-2557. ISSN 0032-9592
79. P. BHOSALE and R. V. GADRE: *β-Carotene production in sugarcane molasses by a Rhodotorula glutinis mutant.* Journal of Industrial Microbiology and Biotechnology, 2001. **26**(6): p. 327-332. ISSN 1367-5435 (print), 1476-5535 (electronic)
80. P. BUZZINI: *Batch and fed-batch carotenoid production by Rhodotorula glutinis–Debaryomyces castellii co-cultures in corn syrup.* Journal of Applied Microbiology, 2001. **90**(5): p. 843-847. ISSN 1364-5072 (print), 1365-2672 (electronic)
81. P. DAVOLI, V. MIERAU, and R. W. S. WEBER: *Carotenoids and fatty acids in red yeasts Sporobolomyces roseus and Rhodotorula glutinis.* Applied Biochemistry and Microbiology, 2004. **40**(4): p. 392-397. ISSN 0003-6838 (print), 1608-3024 (electronic)

82. P. BHOSALE and R. V. GADRE: *Production of ß-carotene by a mutant of Rhodotorula glutinis.* Applied Microbiology and Biotechnology, 2001. **55**(4): p. 423-427. ISSN 0175-7598 (print), 1432-0614 (electronic)

83. A. WEISS, J. DELPROPOSTO, and C. N. GIROUX: *High-throughput phenotypic profiling of gene-environment interactions by quantitative growth curve analysis in Saccharomyces cerevisiae.* Analytical Biochemistry, 2004. **327**(1): p. 23-34. ISSN 0003-2697

84. AMERSHAM BIOSCIENCES: *Gene Navigator™ System. User Manual.* 1997, Uppsala, Sweden: AMERSHAM BIOSCIENCES. 72 p.

85. K. P. RABITSCH, J. GREGAN, A. SCHLEIFFER, J. P. JAVERZAT, F. EISENHABER, and K. NASMYTH: *Two fission yeast homologs of Drosophila Mei-S332 are required for chromosome segregation during meiosis I and II.* Current Biology, 2004. **14**(4): p. 287-301. ISSN 0960-9822